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Canine oral biofilms: cultural, molecular, and *in vitro* studies

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Abstract

The canine oral microbiota is poorly understood compared to that of humans. The aim of this work was to improve understanding of the canine oral microbiota. This was achieved by surveying the canine oral microbiota, determining coaggregation interactions between its members, and developing a laboratory microcosm.

Bacteria were isolated from the dental plaque and saliva of dogs, and isolates were identified by comparative 16S rRNA gene sequencing. From 339 isolates, 84 phylotypes belonging to 37 genera were identified. Approximately half were identified to species level, and 28 % of these were also members of the human oral microbiota. Thirty eight phylotypes were tentatively identified as candidate new species.

The genera most frequently isolated from saliva were *Actinomyces*, *Streptococcus*, and *Granulicatella*. *Porphyromonas*, *Actinomyces*, and *Neisseria* were most frequently isolated from plaque. On average, sequences from this study differed by almost 7 % in the 16S rRNA gene compared to similar organisms from humans.

Targeted PCR was used to detect culture resistant bacteria from canine plaque. Successful amplification indicated that *Spirochaetes* and candidate division TM7 bacteria were present, however the identities of the originating organisms were not determined.

The entire cultivable plaque microbiota from a single dog was assessed for coaggregation reactions. Eight (6.7 %) unique interactions were detected from 120 crosses, indicating that the prevalence of coaggregation is similar in the canine and human oral microbiotas. Genera common to both hosts generally exhibited similar coaggregation reactions, however autoaggregation was more common among bacteria isolated from dogs.

The constant depth film fermenter was used to grow microcosms from canine plaque and saliva using a mucin containing artificial saliva supplemented with horse serum as the growth medium. The model produced biofilms similar to natural dental plaque, which could be used to investigate the canine oral microbiota further.

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Many people have contributed to this work by encouraging and supporting me both academically and personally.

I dedicate this thesis to my wife Pamela, whose continual love and support has been invaluable. Thank you for tolerating my indulgence so happily, and for the sacrifices you have made on my behalf.

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Abbreviations

General abbreviations

AA	anaerobe agar
AS	artificial saliva
BHI	brain heart infusion
bp	base pairs
BPA	black pigmented anaerobic bacteria
CAS	canine artificial saliva
CBA	Columbia agar base
CFAT	cadmium fluoride acriflavin tellurite
cfu	colony forming units
CP	canine plaque
CPP	canine pooled plaque
CPS	canine pooled saliva
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FISH	fluorescent in situ hybridisation
GCF	gingival crevicular fluid
h	hour
PBS	phosphate buffered saline
PCR	polymerase chain reaction
ptype	phylotype
RCF	relative centrifugal force (multiples of g)
RNA	ribonucleic acid
rRNA	ribosomal RNA
S	Svedberg unit (measure of density)
sp.	species (singular)
spp.	species (plural)
Tm	melting temperature
tvc	total viable count
UPGMA	unweighted pair group method with arithmetic means
V	volt (electric potential difference)

Units

m	metre
min	minute
M	moles litre^{-1} (molarity)
MY	million year
MYA	million years ago
y m	year month

Unit prefixes

m	milli- ($\times 10^{-3}$)
μ	micro- ($\times 10^{-6}$)
n	nano- ($\times 10^{-9}$)

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Chapter 1

Introduction

Canis familiaris - the domestic dog, has been a companion of people for at least 14,000 years according to the fossil record (Vilà *et al.*, 2003). We have much in common, including a tendency to suffer from oral ailments such as gingivitis, periodontal disease, and dental caries.

There is a wealth of literature, opinion, and products available for human oral healthcare; so much that most humans do not know what to do with it, however there is little specific information available regarding canine oral healthcare. Humans and dogs are similar in many ways, but it is not necessarily valid to extrapolate basic research or acquired knowledge regarding oral healthcare between these species.

Basic research focused on the dog can help to identify similarities and differences between us, and there are two clear benefits to such work. Firstly, focusing on the dog will promote understanding of the canine condition, providing a sound foundation for improving canine healthcare. Secondly, having data and understanding of our close companion enhances the value of the knowledge already accumulated about humans, by providing data on a model organism for comparison.

This work is a study of the oral microbiota of dogs; the community of microorganisms found in the oral cavity of the dog, in particular the bacteria found in dental plaque and in saliva. Dental plaque is an example of a multispecies biofilm, an intimate association of a mixed microbial consortium attached to the surface of the tooth. Biofilms are also found on the soft tissues of the mouth, but these were not examined in this study.

The canine oral microbiota was characterised by culture-based and culture-independent methods. *In vitro* coaggregation assays were used to detect partner species in canine dental plaque, and a laboratory microcosm model was developed to allow plaque-like biofilms to be reproducibly grown in the laboratory.

1.1 Microbially-mediated oral diseases

The oral cavity of humans and other mammals provides a unique environment, and consequently has a characteristic set of microorganisms which have evolved to exploit it. Unlike other parts of the body, teeth are non-shedding and therefore provide a permanent attachment point for microorganisms. Teeth are anchored in the jaw bone and partially covered by the gums, from which gingival crevicular fluid (GCF) exudes. In addition to GCF, the oral cavity is bathed in saliva and periodically exposed to food. This simplistic description of the mouth is enough to elicit a realisation of the basis for the complex microbial community called dental plaque, which has fascinated microbiologists since the first samples were observed microscopically by Antonie van Leeuwenhoek in the late seventeenth century (Holt, 1978).

Oral diseases include a range of conditions, many of which have a microbial component in their aetiology. It is now well established that the presence of dental plaque is in fact the primary aetiological agent of caries, gingivitis, and periodontal diseases. This has been demonstrated, for example, by germ free rats failing to develop caries (Orland *et al.*, 1954), and by a longitudinal plaque control study in humans showing that removal of plaque, calculus, and diseased tissue effectively halts periodontal disease (Lindhe and Nyman, 1975). Since the importance of dental plaque for oral health was realised, researchers have tried to identify the specific organisms involved in oral conditions, and to understand the mechanisms by which they induce disease or promote health.

Oral diseases may affect the tooth itself, the supporting and surrounding structures of the tooth, or other parts of the mouth such as the cheek and tongue. In addition, oral infections may lead to systemic infections or localised infection at other locations in the body, such as the heart.

Isogai *et al.* (1989a) found the healthy salivary microbiota of dogs to be dominated by *Streptococcus*, *Staphylococcus*, *Lactobacillus*, *Fusobacterium*, and *Veil-*

lonella species. Others have reported that streptococci are uncommon in the canine oral microbiota, but levels of enterococci, *Actinomyces* species and Gram-negative anaerobic rods are considerably higher than levels found in the human microbiota (Wunder *et al.*, 1976). The progression of periodontal disease in the dog is generally reported to be associated with a rise in *Bacteroides* species, *Porphyromonas* species and spirochaetes (for a review see Hennet and Harvey, 1991a).

1.1.1 Periodontal diseases

Periodontal diseases include those conditions which affect the supporting and surrounding tissues of the teeth, in particular the gums and jaw bone. The description of periodontal diseases is relatively complex, covering a continuum of severity, however the most common clinical presentations are gingivitis and periodontitis.

Gingivitis is a reversible inflammation of the gums caused by the activities of microorganisms in dental plaque, and exacerbated by the host immune response. If left unchecked, gingivitis can progress to periodontitis. Periodontitis is a severe inflammation of the gums which results in gingival recession and alveolar bone loss (Figure 1.1) which is irreversible but can be halted by careful oral healthcare. Gingival recession results in the production of a gap between the tooth and the gum called a periodontal pocket, which generates an oxygen depleted nutrient rich niche. Alveolar bone loss results in loss of tooth attachment, and ultimately leads to loss of the affected tooth.

1.1.2 Diseases of the tooth

Coronal caries

Coronal caries is the dissolution of dental enamel by acids produced by the metabolic activities of bacteria in dental plaque. Certain species have been identified as being cariogenic, for example *Streptococcus mutans* is widely regarded as the most important causative agent of caries (Hardie and Whiley, 1999). In general, the microbiota of caries sites is dominated by Gram-positive bacteria, particularly *Actinomyces*, *Lactobacillus*, *Propionibacterium*, and *Streptococcus* species (Munson *et al.*, 2004).

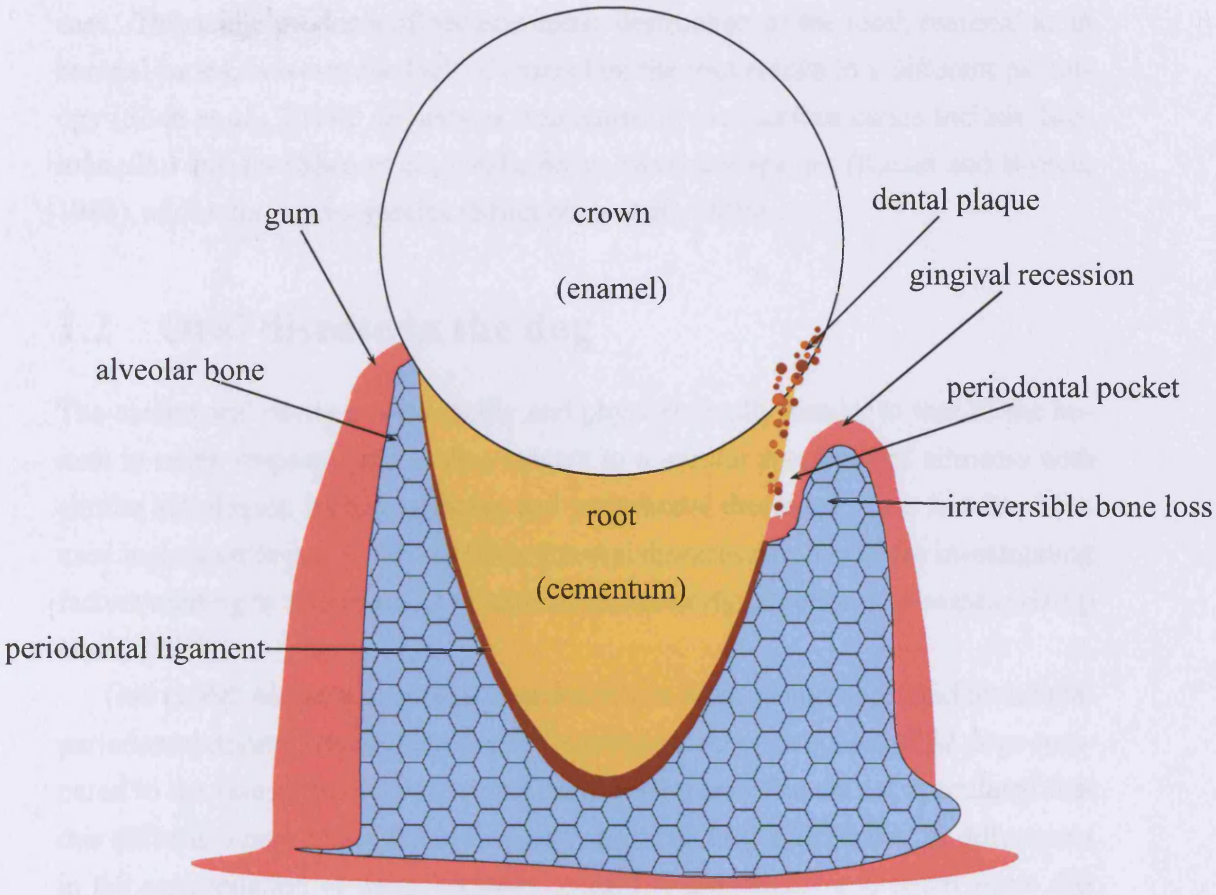


Figure 1.1: Schematic diagram of the tooth and supporting structures. Periodontal disease results in alveolar bone loss and gingival recession, as shown on the right side of the diagram.

Caries is, however, relatively uncommon in dogs, and generally does not result in severe decay (Isogai *et al.*, 1989b). There are several factors which may explain the low prevalence of caries in the dog; the conical shape of the crown, little fermentable carbohydrate in the diet, higher salivary pH, and differences in the oral microbiota (Hale, 1998).

Root surface caries

Root surface caries is a similar phenomenon to coronal caries which may occur when the root surface becomes exposed, for example as a result of periodontal dis-

ease. The acidic products of bacteria cause destruction of the tooth material as in coronal caries, however the lack of enamel on the root results in a different pathology (Shen *et al.*, 2004). Organisms implicated in root surface caries include *Lactobacillus* species (Shen *et al.*, 2004), *Bifidobacterium* species (Kaster and Brown, 1983), and *Actinomyces* species (Schüpbach *et al.*, 1995).

1.2 Oral disease in the dog

The canine oral cavity is structurally and physiologically similar to that of the human in many respects, and is thus subject to a similar spectrum of ailments with similar aetiologies, including caries and periodontal dieseases. This fact has been used in the past to justify the use of canine oral diseases as a model for investigating factors relating to oral diseases of humans, particularly periodontal diseases (Hamp *et al.*, 1997).

Gad (1968) aimed to compare periodontitis in humans and dogs, and found that periodontal destruction occured five times more quickly in a cohort of 62 dogs compared to the rate of periodontal destruction in humans. The author speculated that this difference may be attributed to microbial community differences, or differences in the accumulation of dental deposits related to anatomical and behavioural differences. Nevertheless, it was concluded that the aetiologies of human and canine periodontitis are identical. Although dogs seem to be more susceptible than humans to periodontal diseases, they rarely suffer from caries (Lewis, 1965).

Two later studies, both using the same cohort of 162 dogs, serve to illustrate the incidence and distribution of oral diseases in dogs. In particular they show that animal size, age, and breed are all influencing factors for oral health, and that caries incidence is lower in dogs compared to humans.

Hamp *et al.* (1984) found that periodontitis was common in the cohort, occuring in 54 % of dogs which they report to be similar to the periodontitis incidence in humans. They also noted that periodontitis was found more frequently and with greater severity in smaller dogs compared to larger dogs, in addition the incidence of periodontitis seemed to be breed specific. A radiographic study showed that prevalence and severity of alveolar bone loss resulting from periodontal disease increased with age (Hamp *et al.*, 1997).

Caries incidence was relatively low at 20 %, and this is of particular interest because it is lower than the caries incidence in humans. The authors suggest that the lower caries incidence in dogs may be a consequence of anatomical differences which reduce interdental contact in the dog, higher salivary pH in the dog, or low levels of extracellular polysaccharide producing streptococci in the dog (Hamp *et al.*, 1984).

1.3 Oral microbiota of humans and dogs

Compared to the canine oral microbiota, the human equivalent is very well understood and provides a useful reference for comparison. Figure 1.2 shows a phylogenetic tree based on the 16S rRNA gene sequences of microorganisms found in the human oral cavity. Our understanding of microbial phylogeny has been revolutionised by the use of such comparisons since the potential of the 16S rRNA gene for this purpose was recognised by Woese in 1987. Previous to this revolution, difficult-to-measure phenotypic traits were used to classify microorganisms, resulting in a confused taxonomic scheme based upon artificial classification markers. The 2nd edition of Bergeys Manual embraced the new 16S rRNA based phylogeny and has allowed a much more natural understanding of phylogeny to develop, based upon DNA sequence comparisons (Garrity *et al.*, 2002). The new taxonomic outline recognises two prokaryotic domains; the *Archaea*, and the *Bacteria*. The only *Archaean* genus known to occur in the human oral cavity is *Methanobrevibacter* (Lepp *et al.*, 2004), whose species are strict anaerobes which generate energy by reduction of hydrogen and carbon dioxide to methane. Members of this genus have been associated with periodontal disease by a recent molecular study (Lepp *et al.*, 2004). Some eukaryotes (domain *Eukaryota*), such as the yeast *Candida albicans* or the protozoan *Trichomonas tenax* may also be considered indigenous oral microbiota (Kurnatowska and Kurnatowski, 1998), but members of the domain *Bacteria* are by far the most prevalent microorganisms in the mouth, both in terms of numbers and diversity.

At present the domain *Bacteria* contains 24 recognised phyla (Garrity *et al.*, 2002), of which seven are found in the mouth. Two candidate divisions, TM7 and OP11 are also found in the mouth but have no pure cultured representatives so are

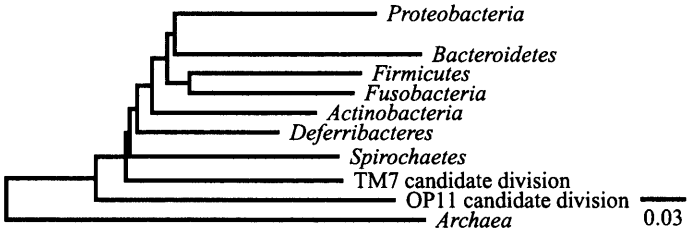


Figure 1.2: Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between bacterial phyla found in the human oral cavity, and the domain *Archaea*. Scale bar indicates 3 % sequence divergence.

not yet recognised as phyla (Paster *et al.*, 2001). The most recent estimates suggest that the oral cavity of humans is home to approximately 500 species of bacteria (Paster *et al.*, 2001), though any single mouth is unlikely to contain such a large number of species. Each of the known oral phyla and their relevant members are discussed below, with reference to their role in human and canine dental plaque.

1.3.1 *Firmicutes*

The *Firmicutes* phylum includes the classes *Clostridia*, *Mollicutes*, and *Bacilli*. The phylogenetic relationships between the organisms of this phylum are shown in Figure 1.3.

Abiotrophia

The genus *Abiotrophia* was defined in 1995 with the recognition that certain nutritionally unusual streptococci did not belong in the genus *Streptococcus*, but should form a separate genus (Kawamura *et al.*, 1995b), which now contains three species; *A. adjaciens, A. defectiva*, and *A. elegans*. There were formerly another three species in the genus which have been re-classified as *Granulicatella* species. *Abiotrophia* species are Gram-positive, catalase-negative cocci which may grow in chains, and are often therefore mis-identified as *Streptococcus* species. Taking this into account, it has been shown that *Abiotrophia* species are among the predominant microorganisms in early dental plaque of humans (Mikkelsen *et al.*, 2000). It was also shown by the same authors that certain *Abiotrophia* strains are able to produce

hydrogen sulphide, which can have various effects upon the local microhabitat and may be considered a virulence factor in periodontal disease.

Catonella

Catonella morbi, the only member of the genus *Catonella*, is an anaerobic Gram-negative bacillus isolated from a human gingival crevice associated with periodontal disease (Moore and Moore, 1994). It has been multiply detected from the human oral cavity in a molecular study (Paster *et al.*, 2001), but no further information regarding this genus is available in the literature.

Centipeda

Centipeda periodontii, the only species of the genus *Centipeda*, is a Gram-negative anaerobic motile bacterium isolated from human periodontal lesions (Lai *et al.*, 1983). Two PCR based studies detected *C. periodontii* in approximately 13 % of subgingival plaque samples but found no correlation with disease state or patient group (Sawada *et al.*, 2000; Siqueira Jr. and Rôças, 2004). One aim of these papers was to identify whether *C. periodontii* is associated with oral disease. However, despite showing no association, they both conclude that further work is required to clear this matter up. This may be tied to the considerable pathogenic potential of this organism, which includes hydrogen sulphide production and immunosuppression, dissuading the authors from concluding that it is innocuous.

Dialister

Dialister pneumosintes was recently described as a suspected periodontal pathogen because a PCR based study of 135 patients showed that the presence of this organism is associated with periodontal disease (Contreras *et al.*, 2000). This genus is closely related to *Veillonella*, and is one of many in the oral cavity that have been overlooked in the past due to isolation and identification difficulties. Its prevalence is now becoming recognised with the advent of molecular analyses, which have led recently to the description of a new species from the oral cavity of humans; *D. invisus* (Downes *et al.*, 2003).

Enterococcus

Enterococci are Gram-positive cocci indigenous to the gastrointestinal tract. *E. faecalis* has been reported to occur in the subgingival plaque of 5% of adult periodontitis patients (Rams *et al.*, 1992), and to frequently occur in root-filled teeth with persisting periapical lesions (Sundqvist *et al.*, 1998).

Eubacterium

This genus contains many phylogenetically unrelated taxa, because it is defined largely on the basis of negative characteristics, and as such has become a convenient genus for placing difficult-to-classify species (Nakazawa *et al.*, 2002). The *Eubacterium* genus consequently contains a diverse group of obligately anaerobic pleomorphic rods, some of which are frequently isolated from periodontitis (Moore *et al.*, 1983; Paster *et al.*, 2001). In addition Kumar *et al.* (2003) recently demonstrated a strong association between *E. saphenum* and chronic periodontitis in a cohort of 66 disease and 66 control subjects using a selective PCR detection method.

Filifactor

The genus *Filifactor* presently contains two species, *F. villosus* (type species), and *F. alocis*, formerly classified as *Fusobacterium alocis* (Jalava and Eerola, 1999) and first isolated from the gingival sulci of patients with periodontal disease (Cato *et al.*, 1985).

Gemella

These Gram-positive cocci may sometimes be mistaken for streptococci, or Gram-negative cocci such as *Neisseria* under the microscope, but they are in fact related to *Abiotrophia* and *Granulicatella* species. The genus presently includes three species; *G. morbillorium*, *G. haemolysans*, and *G. palaticanis* which was first isolated from the oral cavity of a dog (Collins *et al.*, 1999). These organisms are commensals of humans and other animals, but they are also capable of causing serious infections such as endocarditis (Scola and Raoult, 1998).

Granulicatella

The genus *Granulicatella* presently contains only three species, all of which were previously classified as *Abiotrophia* species (Collins and Lawson, 2000), a genus designated to define the nutritionally variant streptococci. *Granulicatella* species are therefore absent from the previous literature concerning canine oral microbiota, and presumably counts for *Streptococcus* and *Abiotrophia* species are correspondingly inflated.

Lactobacillus

Lactobacilli are Gram-positive rods found in small numbers in the human and canine microbiota, and are recognised as an important part of the microbial consortia involved in the aetiology of root surface caries, which also includes *Actinomyces* and *Streptococcus* species (Shen *et al.*, 2004). Lactobacilli are also among the dominant taxa found in dental caries, and are closely related to streptococci which are also heavily implicated in caries (Munson *et al.*, 2004). Even using 16S rRNA gene based molecular methods, distinguishing the species of these genera can be problematic (Munson *et al.*, 2004).

Peptostreptococcus

Peptostreptococci are Gram-positive anaerobic cocci which can be isolated from many sites of the body and are regarded as commensal organisms, though they may participate in polymicrobial or axenic opportunistic infections, especially abscesses. Various species of this genus have also been implicated in root canal infections, gingivitis, and periodontal disease (Riggio and Lennon, 2003). In addition, *P. micros* was identified as a member of the 'orange' complex by Socransky *et al.* (1998), who showed by community ordination using principal components analysis that it associates with a certain group of organisms, indicating a possible link with other bacteria more readily identified as pathogens. *P. micros* has recently been reclassified as the only member in a new genus; *Micromonas micros* (as recorded in a validation list of the International Journal of Systematic and Evolutionary Microbiology, 50:1415-17, 2000).

Pseudoramibacter

Pseudoramibacter alactolyticus was formerly classified as *Eubacterium alactolyticum*, it is the only species of the genus *Pseudoramibacter* and has been isolated from various oral sites (Downes *et al.*, 2001).

Selenomonas

Selenomonas species are Gram-negative rods commonly found in water systems but have also been identified as being associated with periodontitis (Dzink *et al.*, 1988; Tanner *et al.*, 1998) and are often identified from oral samples.

Solobacterium

Solobacterium species belong to the class *Mollicutes* which are rare in the oral cavity, but *S. moorei* has been detected in oral samples by two recent molecular studies (Paster *et al.*, 2001; Rolph *et al.*, 2001).

Staphylococcus

Staphylococci may be detected in the oral microbiota, but are generally regarded as transient contaminants from the skin microbiota rather than indigenous species.

Streptococcus

The streptococci include primary colonisers of the human tooth surface such as *S. sanguinis* (formerly *S. sanguis*), and acidogenic species such as *S. mutans*, which are the primary causative agents of dental caries in humans (Hardie and Whiley, 1999). They are a diverse and abundant group of Gram-postitive cocci in the human oral microbiota, typically comprising approximately 28 % of the cultivable microbiota in mature dental plaque (Socransky and Manganiello, 1971), and up to 85 % in early dental plaque (Nyvad and Kilian, 1987).

The high incidence of streptococci in early dental plaque highlights their important role in the primary colonisation of the tooth surface. After teeth are cleaned they quickly acquire a complex film of salivary components of bacterial and host origin called the pellicle. Streptococci can adhere to many components in the acquired

pellicle, including acidic proline-rich-proteins, alpha-amylase, and sialic acid (for a review see Whittaker *et al.*, 1996).

After colonisation, the pioneer streptococci facilitate further development of the plaque by acting as a receptor surface to which secondary colonisers must adhere if the plaque is to develop further. An unusual feature of the streptococci shown by Kolenbrander *et al.* (1990), is that they commonly have partners of the same genus to which they specifically adhere (intra-generic coaggregation). Apart from a few *Actinomyces* species which are also primary colonisers, this property is unique in the human oral microbiota. The authors proposed that this feature is probably an adaptation favouring the primary colonisation of the tooth surface.

In addition to their structural and developmental significance in dental plaque, streptococci are also metabolically important, and attract particular attention because of their prominent role in the aetiology of dental caries. Of the cariogenic species, *S. mutans* seems to be the most important, but *S. sobrinus* also plays a significant role (Colby and Russell, 1997). These organisms produce acid by the fermentation of sugars in the diet, and frequent acidic conditions may ecologically disrupt the plaque microbiota by selecting for an aciduric community (Marsh, 2003).

Streptococci do not seem to be of such great importance in canine dental plaque, as evidenced by frequent reports of low numbers of streptococci in canine investigations. For example, Wunder *et al.* (1976) found that streptococci comprised less than 4 % of the supragingival plaque of two beagle dogs, and Syed *et al.* (1981) noted that *S. sanguis* and *S. mutans* were not found in canine dental plaque. In addition, dogs rarely suffer from caries (Lewis, 1965) so cariogenic species in dogs do not have the economic significance of cariogenic species in humans.

Veillonella

Veillonella species are anaerobic Gram-negative cocci resident in the oral cavity, especially in dental plaque where they metabolise lactic acid produced by other bacteria, producing weaker propionic and acetic acids. Following the finding that the presence of *Veillonella* species in an *in vitro* model increased the acid-producing capacity of *S. mutans* (Noorda *et al.*, 1988), and an association with carious lesions (Becker *et al.*, 2002), it has been suggested that *Veillonella* species may play an

important role in caries by protecting acid producing species, however by the same mechanism it is also possible that they may reduce the cariogenic potential of acid producing species.

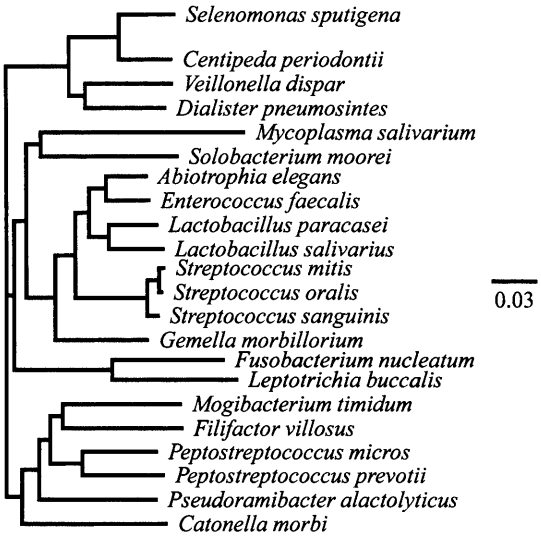


Figure 1.3: Phylogenetic tree showing the relationship between species of the phyla *Firmicutes* and *Fusobacteria* found in the oral cavity. Scale bar indicates 3 % sequence divergence.

1.3.2 *Fusobacteria*

The *Fusobacteria* phylum is relatively small, and has only two genera commonly found in the oral cavities of humans and dogs, *Fusobacterium*, and *Leptotrichia*. The phylogenetic relationships between the organisms of this phylum and the *Firmicutes* phylum are shown in Figure 1.3.

Fusobacterium

Fusobacterium is an important genus of Gram-negative anaerobic rods; they typically form long filaments and are able to co-aggregate with most other oral bacteria, which is thought to give them an important structural role in dental plaque (Kolenbrander *et al.*, 2002). *Fusobacterium* species are also involved in the production of

odours in halitosis due to their metabolism of sulphur-containing compounds (Lee *et al.*, 2004)

Leptotrichia

The human oral cavity has been called the primary habitat of this genus which contains several species of large fusiform rods (Eribe *et al.*, 2004), but presumably it is also indigenous to the mouths of other animals. *L. buccalis* is common in the subgingival dental plaque of humans in health and disease (Paster *et al.*, 2001).

1.3.3 *Actinobacteria*

The *Actinobacteria* are common in the human oral microbiota, for example making up over 50 % of anaerobic isolates from dental caries (Munson *et al.*, 2004). However, the same study confirmed that the phylum was under-represented by molecular analyses, probably as a result of the high G+C content in the phylum reducing the efficiency of the *Taq* polymerase used in PCR.

The phylogenetic relationships between the organisms of the phylum *Actinobacteria* and the phyla *Deferribacteres* and *Spirochaetes* are shown in Figure 1.4.

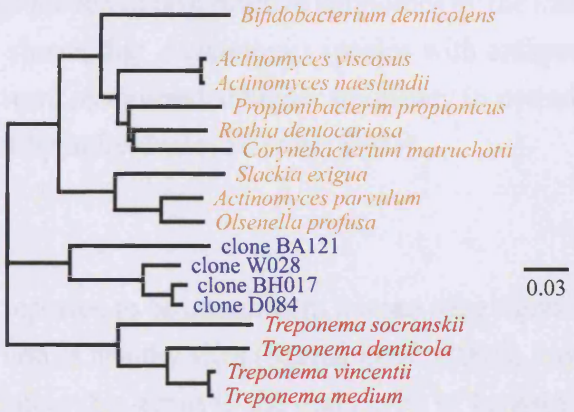


Figure 1.4: Phylogenetic tree showing the relationship between species of the phyla *Actinobacteria*, *Deferribacters*, and *Spirochaetes* found in the oral cavity. Scale bar indicates 3 % sequence divergence.

Bifidobacterium

Bifidobacterium species are common in the human intestinal tract (Matsuki *et al.*, 1999), but they are also sometimes found in the oral cavity at both subgingival sites (Paster *et al.*, 2001) and supragingival sites (Crociani *et al.*, 1996) where they have been found in association with dental caries. Isolates which produce extracellular endohydrolytic dextranases have also been found in association with root surface caries, and these organisms were able to utilise the dextran degradation products, producing acid in the process (Kaster and Brown, 1983).

Actinomyces

Most *Actinomyces* species are indigenous to the mucous membranes of humans and animals, particularly in the oral cavity (Hoyles *et al.*, 2000). They are abundant Gram-positive rods in human dental plaque, and are implicated in gingivitis and root surface caries (Schüpbach *et al.*, 1995). Similarly, *Actinomyces* species are abundant in canine dental plaque, and along with streptococci they have been reported to be particularly involved in primary colonisation of the tooth (Hennet and Harvey, 1991c).

A possible mechanism by which bacteria may induce inflammation of the gingiva is if they elicit the production of host reactive antibodies by the immune system, and recent evidence has shown that *Actinomyces* species with antigens cross reactive with epithelial cells were recognised at higher frequency in periodontal disease patients compared to healthy individuals (Ye *et al.*, 2003).

Corynebacterium

C. matruchotii has been reported to be common in human subgingival samples, but is apparently more common at healthy sites (Kumar *et al.*, 2003), however there is little further literature relating this genus to the oral cavity of humans. *C. kutscheri* is reported in the oral microbiota of various small animals, including rats, ferrets, and hamsters (Amao *et al.*, 2002; Fischer *et al.*, 1994; Amano *et al.*, 1991).

Propionibacterium

There is very little literature available concerning *Propionibacterium* in the oral cavity, however it is occasionally detected (e.g. Paster *et al.*, 2001).

Rothia

R. dentocariosa has been found to be the second most prevalent organism in throat cultures of healthy individuals, being isolated in 29 % of cases from 113 people (von Graevenitz *et al.*, 1998). In addition, *R. dentocariosa* has been found to be negatively correlated with periodontal pocket depth and the presence of black-pigmented bacteria in subgingival samples, indicating that it may play a protective role in the host (Tanner *et al.*, 1979).

Mogibacterium

Mogibacterium species are Gram-positive anaerobic rods, all five of which have been isolated from the human oral cavity, particularly in association with periodontal disease and infected root canals. These bacteria are phenotypically similar to *Eubacterium* species, hence the transfer of *E. timidum* to *M. timidum* at the time of the new genus proposal in 2000 (Nakazawa *et al.*, 2000).

Slackia

The genus *Slackia* was proposed in 1999 (Wade *et al.*, 1999) to accommodate *Eubacterium exiguum* and *Peptostreptococcus heliotrinreducens*, which were shown by comparative 16S rRNA gene sequencing and phenotypic characterisation to merit transfer. These Gram-positive anaerobic bacilli are now called *S. exigua* and *S. heliotrinreducens* respectively, and as with many of the present and former *Eubacterium* species, they are difficult to identify phenotypically, however *S. exigua* is often found in periodontitis and periapical infections (Wade *et al.*, 1999).

Olsenella

Lactobacillus uli is a strong producer of lactic acid originally isolated from human gingival crevices and periodontal pockets (Olsen *et al.*, 1991), and was reclassified

in 2001 under the new genus *Olsenella* as *O. uli*, along with *O. profusa* (Dewhirst *et al.*, 2001). The strains on which the description for this latter species was based were previously designated as *Eubacterium* species and came from the large culture collection of Moore and Moore in the 1970's. Recently, a culture-based study on using root canal samples from apical periodontitis sites found that of the Gram-positive bacteria, 38 % were *Lactobacillus* species and 18 % were *O. uli* (Chávez de Paz *et al.*, 2004), however there is little further information regarding this genus in the current literature.

1.3.4 *Spirochaetes*

A relationship between *Spirochaetes* and periodontal disease has been recognised for a long time. This is perhaps in part due to the distinctive helical morphology and motility of spirochaetes making them easy to detect microscopically, despite their fastidious requirements making them very difficult to detect by culture. Microscopically, *Spirochaetes* frequently make up a large proportion of the subgingival microbiota both in humans (Dahle *et al.*, 1993) and dogs (Hennet and Harvey, 1991b). At present, all identified oral *Spirochaetes* belong to the genus *Treponema*, of which there are over 60 known species or phylotypes (Paster *et al.*, 2001), only four of which have been reliably maintained by culture (Chan and McLaughlin, 2000).

1.3.5 *Proteobacteria*

In their analysis of 2,522 clones from subgingival plaque, Paster *et al.* (2001) detected 28 known species and 23 novel phylotypes of *Proteobacteria*, most of which were found only sporadically.

Gram-negative rods found in the human oral microbiota include *Haemophilus* species and *Eikenella corrodens*, which are facultatively anaerobic, and *Capnocytophaga* and *Actinobacillus* species which are capnophiles. While *Haemophilus* species are not implicated in disease, *Eikenella*, *Capnocytophaga* and *Actinobacillus* species are implicated to varying degrees in gingivitis and periodontitis.

The phylogenetic relationships between the organisms of the phylum *Proteobacteria* are shown in Figure 1.5.

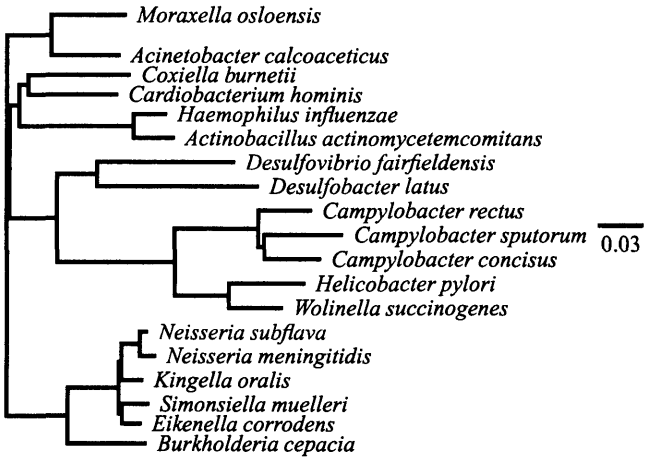


Figure 1.5: Phylogenetic tree showing the relationship between species of the phylum *Proteobacteria* found in the oral cavity. Scale bar indicates 3 % sequence divergence.

Actinobacillus

A. actinomycetemcomitans was transferred to the genus *Haemophilus* in 1985 (Potts *et al.*, 1985) but the validity of this transfer has been disputed (e.g. as recorded in the minutes of a meeting of the International Committee on Systematic Bacteriology meeting published in the International Journal of Systematic Bacteriology, 37:474, 1987). At present it is technically correct to refer to the organism as *Haemophilus actinomycetemcomitans*, however many workers, and the present work, continue to use *Actinobacillus actinomycetemcomitans*.

A. actinomycetemcomitans can be isolated from many oral sites in both periodontally healthy and diseased individuals, and is a member of the indigenous human oral microbiota (Henderson *et al.*, 2003). This organism is heavily implicated in the pathogenesis of localised aggressive periodontitis in humans (Slots, 1999), in which a variety of virulence factors seem to be involved. However, this organism is rarely detected in the canine oral cavity (e.g. Allaker *et al.*, 1997a).

Cell surface fibrils on fresh clinical isolates are associated with the ability to adhere and form biofilms on oral surfaces, and this feature is sometimes lost upon successive laboratory culture (Henderson *et al.*, 2003)

Campylobacter

Campylobacter species are best known for their causative role in bacterial gastroenteritis which can be due to several different species, *C. jejuni* being by far the most common (Butzler, 2004). Certain *Campylobacter* species have also been found in the oral microbiota; for example by the statistical clustering analyses performed by Socransky *et al.* (1998) on DNA-DNA checkerboard hybridisation data from 13,261 plaque samples. These workers found that *C. rectus* (formerly *Wolinella recta*; Vandamme *et al.*, 1991), *C. showae*, and *C. gracilis* were often associated with *F. nucleatum*, *Prevotella* spp., and *Micromonas micros*, in what they termed the 'orange' cluster. Although the relationship of this cluster to health status was not addressed directly, members of the 'red' cluster (*T. forsythus*, *P. gingivalis*, and *T. denticola*) which was significantly associated with periodontal disease were rarely found in the absence of 'orange' cluster species. Paster *et al.* (2001) found that *C. rectus* was associated with samples from periodontal disease, whilst *C. gracilis* and *C. concisus* were found in samples from healthy and diseased sites. In addition, a recent study into periodontal risk factors in 107 Japanese high school students found that *C. rectus* was present in 89 % of students, and its proportion was significantly related to clinical parameters (Suda *et al.*, 2004).

Cardiobacterium

C. hominis and the recently described *C. valvarum* are the only members of the genus *Cardiobacterium* (Han *et al.*, 2004). *C. hominis* is detected in the oral microbiota occasionally (Paster *et al.*, 2001) but is rarely discussed in any detail by the present literature so its role in the oral cavity remains obscure; rather this genus gains attention because it causes bacterial endocarditis, and a likely source of infection in such cases is dental plaque (Lockhart, 2000).

Desulfobacter and *Desulfovibrio*

The first to show that sulphate-reducing bacteria reside in the human oral cavity were van der Hoeven *et al.* (1995). Using enrichment cultures they found sulphate-reducing bacteria in the subgingival plaque of 58 % of 43 individuals tested, and

pure isolates of the genera *Desulfobacter* and *Desulfovibrio* were found to be among them.

Eikenella

Eikenella corrodens is a facultative anaerobe and the only member of the genus *Eikenella*. It has been associated with various non-oral infections, and is commonly found in the oral cavity of healthy and periodontally diseased individuals (Fujise *et al.*, 2004; Chen and Wilson, 1992). Fujise *et al.* (2004) investigated the clonal diversity of subgingival *E. corrodens*; they found that many subjects harbour multiple clones and that clonal diversity was higher in periodontal disease compared to healthy subjects. Clonal diversity and clonal shifts over time were suggested by these authors as possibly indicating that *E. corrodens* can colonise subgingival sites more easily than many other bacteria; in any case such observations highlight the complexity of the oral microbiota.

Haemophilus

Haemophilus species are sometimes reported in the oral cavity of humans, and have been found to harbour amoxycillin resistance in the dental plaque of children (Ready *et al.*, 2004).

The closely related organism *Actinobacillus actinomycetemcomitans* technically belongs to the genus *Haemophilus*, though biologically it may deserve to be in a separate genus (see Section 1.3.5). Reports of *Haemophilus* species in the canine oral cavity are rare.

Helicobacter

Helicobacter pylori is well known for its role in gastric disease, but it has also been detected in plaque and saliva both by culture (Cześnikiewicz-Guzik *et al.*, 2004) and by PCR (Gebara *et al.*, 2004). The role of this organism in the mouth, if any, is unclear, but it seems that the oral cavity can act as a reservoir for this gastrointestinal pathogen.

Kingella

K. oralis is common in the oral cavity, and it may easily be mistaken for *Eikenella corrodens*, having similar phenotypic traits and distribution, though there is no evidence to link this organism with the aetiology of periodontal disease (Chen, 1996).

Moraxella

Moraxella catarrhalis, formerly *Branhamella catarrhalis* and *Neisseria catarrhalis*, often behaves as an upper respiratory tract commensal, and is difficult to distinguish from *Neisseria* species sharing the same habitat, however it has become recognised as an important cause of respiratory tract infection (Greiner *et al.*, 2003).

Neisseria

Neisseria species are often isolated from the oral cavity of humans, but there is little literature concerning their role, indicating that they are rarely implicated in disease. A notable species of animal origin is *N. dentiae*, first isolated from the dental plaque of a cow by Sneath and Barrett (1996), who suggest this organism may play a significant role in the generation of an anaerobic microenvironment due to their rapid consumption of oxygen.

Simonsiella

The short flat cells of the genus *Simonsiella* form filaments and display gliding motility, and have been described as oral commensals of mammals (Hedlund and Staley, 2002). They have been reported in the oral cavities of many animals, including cats, dogs, sheep, and humans (Kuhn *et al.*, 1978). Surprisingly, the incidence of this relatively unknown genus in the oral cavity of dogs was assessed in 1977; Nyby *et al.* (1977) found *Simonsiella* species in 66 of 67 dogs by swabbing the palate, and came to the obvious conclusion that these bacteria are residents of the canine oral cavity. There are few reports of *Simonsiella* species in the oral cavity of humans and it has been suggested that they may be merely transient inhabitants (Carandina *et al.*, 1984); this is supported by the fact that none were detected by the exhaustive cloning excercise performed on subgingival plaque by Paster *et al.*

(2001), however they may be inhabitants of the soft tissues as they appear to be in dogs, or they may be incompatible with the cloning procedure employed by Paster *et al.*.

Wolinella

Wolinella recta has been associated with periodontal disease (Lai *et al.*, 1992), but its role in the disease process is not clear. The complete genome sequence of *W. succinogenes* has been obtained due to it's similarity to the pathogens *Helicobacter pylori*, and *Campylobacter jejuni* (Baar *et al.*, 2003).

1.3.6 *Bacteroidetes*

A large proportion of the oral microbiota is composed of anaerobic Gram-negative rods including the genera *Porphyromonas* and *Prevotella*. These genera are heavily implicated in periodontal diseases, especially *Porphyromonas gingivalis* in humans, which attracts a great deal of research and is found almost exclusively at subgingival sites, particularly those associated with periodontal disease. Several *Capnocytophaga* species are found in the oral cavity where they may be implicated in disease.

The phylogenetic relationships between the organisms of the *Bacteroidetes* phylum are shown in Figure 1.6.

Bergeyella

Bergeyella zoohelcum is an uncommon zoonotic pathogen typically associated with cat or dog bites, it is the only representative of the genus *Bergeyella*, and was formerly called *Weeksella zoohelcum*.

Capnocytophaga

Capnocytophaga species are indigenous to the human oral cavity both in health and disease, and they are considered putative periodontal pathogens in light of their various virulence factors and isolation from a range of periodontal disease samples (Ciantar *et al.*, 2001).

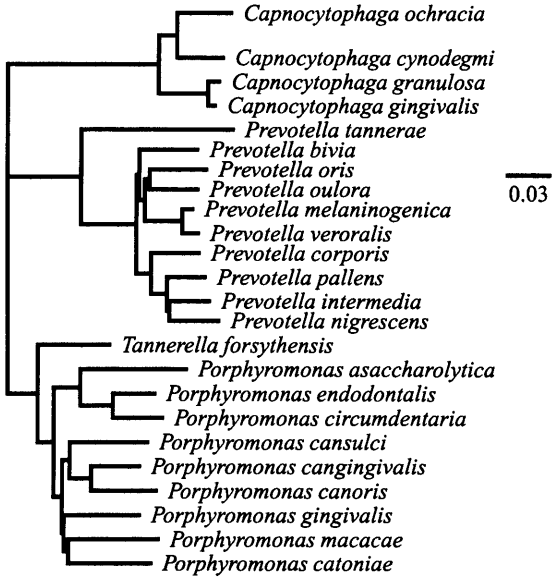


Figure 1.6: Phylogenetic tree showing the relationship between species of the phylum *Bacteroidetes* found in the oral cavity. Scale bar indicates 3 % sequence divergence.

Porphyromonas

Bacteroides gingivalis has been established as a periodontal pathogen for many years due to it's frequent isolation from periodontal disease related samples (e.g. Socransky *et al.*, 1988), and it has therefore received much attention from researchers.

In 1988 *B. gingivalis* was reclassified into the new genus *Porphyromonas* (Shah and Collins, 1988) as *P. gingivalis*. The *Porphyromonas* genus was proposed to accommodate the asaccharolytic *Bacteroides* species, and there are several species of this fastidious anaerobic genus found in the oral cavities of humans and animals. *P. gingivalis* has been reported numerous times in the canine oral microbiota, but with the assistance of 16S rRNA gene based phylogenetics it is becoming clear that isolates from dogs are often a different species, usually *P. gulae* which is distinguished from *P. gingivalis* by its positive reaction to the catalase test (Fournier *et al.*, 2001).

Porphyromonas species are common in the dog and have been associated with periodontal disease (Allaker *et al.*, 1997b; Isogai *et al.*, 1999), suggesting that they may play a similar pathogenic role in dogs and humans.

Prevotella

The *Prevotella* genus accommodates saccharolytic organisms formerly belonging to the *Bacteroides* genus. Two species of *Prevotella* are frequently reported in human plaque samples, *P. intermedia*, and *P. nigrescens* (e.g. Moore *et al.*, 1987); whilst the former may be considered a periodontal pathogen, the presence of the latter may be a marker for relative periodontal health (Gmür and Thurnheer, 2002). *P. intermedia* has been found to commonly reside in the canine oral cavity, and *P. denticola* was found to be present occasionally (Allaker *et al.*, 1997b).

Tannerella

Tannerella forsythensis was formerly classified as *Bacteroides fusiformes*, then *Bacteroides forsythus*, until recently the genus *Tannerella* was proposed to accommodate it (Sakamoto *et al.*, 2002). *T. forsythensis* is a fastidious fusiform found in periodontal pockets of humans (Tanner *et al.*, 1979), and is now recognised as an important periodontal pathogen (Genco, 1996), and it has been suggested that recognition of this was delayed due to it's fastidious growth requirements in the laboratory (Sanz *et al.*, 2004).

1.3.7 *Deferribacteres*

The *Deferribacteres* phylum has few cultured representatives and it has never been isolated from the oral cavity, however a culture-independent study has identified two clones associated with chronic periodontitis, and another associated with periodontal health (Kumar *et al.*, 2003; Paster *et al.*, 2001). A total of eight *Deferribacteres* phylotypes were detected in subgingival plaque samples by PCR, cloning, and sequencing, indicating that members of this phylum are previously-overlooked indigenous oral microbes of humans.

1.3.8 Candidate divisions TM7 and OP11

The candidate divisions TM7 and OP11 have no cultivated representatives, but they have been identified by numerous molecular rRNA based studies which indicate a great diversity of phylotypes (Hugenholtz *et al.*, 2001; Harris *et al.*, 2004). The

sequence diversity of the candidate division OP11 is so great that recent analyses have shown that it should be split into three candidate divisions, which themselves are highly diverse Harris *et al.* (2004). Members of TM7 and OP11 have been detected in dental plaque, and a clone of each has been associated with chronic periodontitis in humans; TM7 clone I025 and OP11 clone X112 (Kumar *et al.*, 2003; Paster *et al.*, 2001).

1.4 Modelling the oral microbiota

Using natural samples to investigate the microbial diversity and disease associations of oral biofilms has provided a sound foundation for understanding oral diseases. For example by taking samples from patients presenting different clinical symptoms and determining the bacteria present, it has been possible to link certain species to disease status. Such a link implicates the organism in the disease process, but it is often not clear whether this indicates a causative role or a benign tendency to proliferate in the diseased area.

Koch's postulates (reviewed by Fredericks and Relman, 1996) state that if an organism can be isolated from a diseased site, and upon inoculation into a healthy host replicate the disease, then the organism can be said to cause the disease, providing that also the parasite occurs in every case of the disease and is not found in other diseases as a nonpathogenic parasite. Koch's insight has served microbiologists and clinicians well, but his postulates can not be easily applied to polymicrobial conditions such as periodontal diseases, which may be caused by the synergistic activity of many different combinations of organisms. Despite this, experiments with germ-free animals have been able to link certain bacteria with periodontal diseases, and such experiments can be considered the most basic or the most complex laboratory models available depending upon one's point of view.

The purpose of laboratory models is to provide reliable representations of natural systems which can be manipulated and sampled in such a way that confounding factors can be eliminated from a study, to allow the researcher to vary ideally just one parameter at a time. Such systems allow cause and effect to be easily separated, and may be set up by a large variety of techniques.

Models can be broadly categorised as *in vitro* or *in vivo* systems, depending

on whether they are artificial or animal-based respectively. *In vitro* models are of greatest interest in the present work, one aim of which was to develop such a system for modelling the oral microbiota of dogs, to allow the undertaking of detailed investigations into biofilm systems of relevance to canine oral health without causing suffering to dogs.

In vitro models can be broadly categorised into three types depending upon the inoculum used, which may be either a single species, a defined consortium, or a natural sample (e.g. plaque). Attempts to understand plaque from studies on monocultures have previously been described as heroic (Sissons, 1997), in recognition of the fact that dental plaque is strictly polymicrobial, though monoculture studies certainly have some value for investigation of very specific interactions.

Systems inoculated with plaque or saliva are often referred to as microcosm systems, which have been defined by Wimpenny (1988) as 'Laboratory models of the natural system from which they originate but from which they also evolve'. The natural evolution of microcosm systems is both their strength and their weakness; on the one hand microcosm systems offer the possibility of the most realistic laboratory models, but on the other their realism can result in poor reproducibility and analytical complexity reminiscent of the natural systems they model.

Ideally of course, we would like the laboratory model to be similar to the natural system from which it originated; by comparison with the natural system we can assess the usefulness of the microcosm as a model of the natural system.

1.4.1 *In vivo* systems

In vivo models of dental plaque have been used extensively in dental research, often using the dog as the model organism. Typically, the oral health of the dog is deliberately compromised in some way and the investigator attempts to determine relevant changes associated with the development of disease. For example, ligatures may be placed around the teeth whilst feeding the animal on a soft diet to promote plaque accumulation and the development of periodontal disease (Nociti *et al.*, 2001). Other studies have infected gnotobiotic animals with suspected periodontal pathogens to investigate the link between bacteria and clinical parameters (Orland *et al.*, 1954).

In vivo studies may not be suitable in many cases due to the inherent difficulty of generating reproducible conditions, their high cost, or because of ethical concerns.

1.4.2 Planktonic *in vitro* systems

Batch culture

Batch culture of microorganisms can be used to investigate microbial physiology, or to obtain high cell densities for other studies, however the planktonic growth phase and high nutrient concentrations employed for batch culture are unlikely to accurately model oral biofilms.

Continuous culture

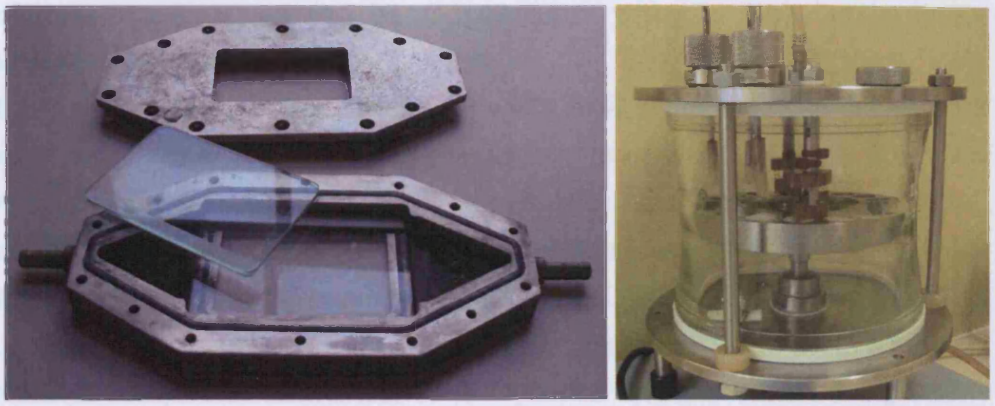
It is possible to control continuous cultures to maintain a predetermined growth rate, which may be governed by nutrient limitation to simulate various scenarios. Despite gross chemical similarities, however, the planktonic mixed culture is unable to replicate the biofilm structure of dental plaque. This simplification may be useful in some cases, and many interesting interactions have been identified using such systems.

Marsh *et al.* (1983) showed that the chemostat could stably support diverse communities of oral bacteria, contrary to usual chemostat dynamics in which mixed cultures are displaced by a a single organism with the strongest competitive advantage, highlighting the importance of inter-bacterial co-operation in dental plaque. Later, Bradshaw *et al.* (1994) used defined communities of oral bacteria to demonstrate that by metabolic co-operation, more diverse communities have higher overall viable cell numbers and are likely to have greater ecological stability.

1.4.3 Biofilm *in vitro* systems

Biofilm models are considered generally to be the most useful systems for modelling dental plaque due to the fact that dental plaque is a biofilm. It is widely recognised that the properties of biofilms are greater than the sum of their planktonic parts, therefore biofilm models are to be preferred where possible. For example, attachment to a surface can induce phenotypic changes (Otto and Silhavy, 2002), the

close association of cells in a biofilm may induce changes through quorum sensing mechanisms (Sauer and Camper, 2001), and the physical structure of the biofilm has many implications for the bacteria belonging to it; for example oxygen and nutrient concentrations are likely to vary throughout the biofilm, and organisms in physical proximity to each other are likely to participate in metabolic cooperation or competition. Interestingly, contrary to early predictions and common sense, the high resistance of biofilms to antimicrobial compounds seems in some cases not to be explained by reduced penetration or increased quenching (Anderl *et al.*, 2000), though these mechanisms may play a role in other cases (Stewart, 2002).



(a) Flow cell. (b) CDFF.

Figure 1.7: Photographs showing two models used for growing *in vitro* biofilms; the parallel plate flow cell and the constant depth film fermenter (CDFF).

Flow cell

The flow cell (Figure 1.7a) typically consists of two parallel glass plates enclosing a chamber through which a liquid culture medium is passed, to provide a nutrient source to bacteria adhering to the glass plates. An optically-clear hydroxyapatite coating on glass has recently been demonstrated for modelling the tooth surface by Elliott *et al.* (2005), and this allows the investigator to use a more biologically-relevant substratum material than glass. Flow cells allow continuous non-destructive observation of biofilm development by microscopy (Busscher and van der Mei, 1995), and are ideally suited for short term adhesion and colonisation

studies. The biofilm may also be sampled at the end of an experiment by dismantling the apparatus.

Modified Robbins device

The Robbins device, modified by McCoy *et al.* (1981) is essentially similar to a flow cell, consisting of a flow chamber with removable substratum samples fitted flush on the interior so that they do not interrupt fluid flow. Aseptic removal of biofilm samples allows replicates and sequential samples to be taken for analysis, something which can not be done with a conventional flow cell. There is no provision for real-time microscopic examinations to be made as there is with a flow cell, but the device could conceivably be modified to allow this.

By their very nature, flow chamber models such as the Robbins device lend themselves well to modelling aqueous systems with defined laminar or turbulent flow characteristics, such as dental unit water lines and catheters. As for modelling dental plaque, flow systems can be used and seem particularly well suited to studying attachment and related phenomena, especially with simple defined communities (e.g. Busscher *et al.*, 1992). Their capacity for generating complex plaque-like microcosms is likely to be limited due to the fact that they provide a homogeneous environment and are fully submerged.

Perfusion

Perfusion systems can be described as those in which the medium is passed through a porous support which serves as a substratum for the biofilm. An example of this is the device developed by Gilbert *et al.* (1989), which consisted of a cellulose acetate membrane through which sterile growth medium was passed to support biofilm growth on the membrane. The great advantage of such systems is that growth rate can be controlled by the rate of addition of growth medium in a similar fashion to that used in the chemostat.

The cellulose acetate membrane perfusion system has been further developed by replacing the membrane with Sorbarod filters which consist of a concertina of cellulose fibres (Hodgson *et al.*, 1995); this modification simplifies operation and increases the biomass of the system. A further recent modification incorporated multi-

ple Sorbarod filters in the same perfusion chamber to provide replicate biofilms, and this system has been used to successfully grow reproducible salivary microcosms (McBain *et al.*, 2005).

CDFF

The constant depth film fermenter (CDFF, Figure 1.7b) is a biofilm growth device designed to allow biofilms to reach a steady state by mechanically maintaining a constant volume (Wimpenny *et al.*, 1993). The system essentially consists of a rotating turntable into which replicate discs of substratum material are recessed. Growth medium drips onto the rotating turntable, the top of which is scraped by static blades which serve to distribute the growth medium and limit the biofilm thickness to the pre-determined depth.

The CDFF was first applied to modelling dental plaque by Wilson *et al.* (1995), and later by Kinniment *et al.* (1996) who used a nine membered bacterial community grown to steady state in an anaerobic chemostat as an inoculum for the CDFF which was operated under aerobic conditions. Despite being open to atmospheric gases, the CDFF developed steady state biofilms which were numerically dominated by the strict anaerobe *Porphyromonas gingivalis*. The climax communities attained after about 400 hours were found not to be the same between runs, and the authors suggested that there may be a number of different states able to satisfy the demands of the system. This seems likely to be the case and is an important issue to consider when comparing experimental data between runs. It seems inevitable that the oral cavity itself would also be subject to the same potential for alternative ecologies.

1.5 Methods of community analysis

The purpose of laboratory models is to provide samples produced in a controlled fashion for subsequent analysis and interpretation to provide data relevant to the system being modelled. In the case of oral biofilm models, samples are most likely to resemble dental plaque or saliva, and the chosen methods of analysis used are likely to be the same as the methods used for natural samples.

1.5.1 Microscopic

Overview

Microscopic analyses can quickly give the investigator all kinds of information about the physical structure of a sample, and allow counting or identification of physically distinct structures such as different cell shapes or Gram-stain reactions. In the absence of physically-distinguishable features, a vast array of staining techniques can be used to selectively mark features of interest. For many years the use of simple staining procedures such as the Gram-stain, acid fast stain, and capsule stain have allowed often undefined features of bacterial cells to be the basis of taxonomic differentiation. Such methods remain useful to this day, however they are generally discovered by trial and error and limited to discrimination of gross structural differences.

Staining and probe targeting

More sophisticated methods of staining have allowed microscopy to remain a powerful method in modern microbiology, in particular the use of antibodies or oligonucleotide probes conjugated to various markers. Antibodies can be used to target specific antigens exposed in a sample, particularly cell surface antigens, however care must be taken to ensure that cross reactivity with other antigens in the sample does not occur. The chance of this can be greatly reduced if monoclonal antibodies are raised against purified proteins. For example Elliott *et al.* (2003) successfully labelled the CshA fibrils of various *Streptococcus* species using monoclonal antibodies raised against the purified heterologous protein, confirming the cell surface location of these adhesins and allowing them to be distinguished from other structurally similar features.

In keeping with the modern obsession with molecular biology, microscopy too has 'gone molecular' since the advent of fluorescent *in situ* hybridisation (FISH) in 1989 (DeLong *et al.*, 1989). This technique exploits the base-pairing property of nucleic acids, allowing the investigator to develop nucleic acid probes which will specifically bind to other nucleic acids of complementary base sequence. Probes used in microbiology are typically oligonucleotides directed at the structural RNA

in ribosomes, but it is theoretically possible to target any nucleic acid in the cell. There are two strong advantages to the ribosomal target though; the large copy number of targets per cell, and the fact that rRNA gene sequences have become the sequence of choice for rapid identification by comparative gene sequencing. FISH can therefore be used to identify bacteria from environmental or laboratory samples to the species level with individual cell resolution under the microscope, and this technology has been extensively applied to samples of oral origin, both *in vitro* and *in vivo*.

Types of microscopy and their limitations

Resolving power in conventional microscopes is limited by the wavelength of light used to view the specimen; put simply, the illuminating wavelength must be smaller than the distance between two objects or they will be seen as one. Confocal laser scanning microscopy (CLSM) is also limited by the wavelength of light but by using a focused scanning laser beam coupled with a sensitive detector, it is possible to extract depth information from the sample which is not possible using transmission or reflection of light in conventional microscopes.

The electron microscope overcomes the light wavelength limitation by using an electron beam instead of a light beam to examine the sample, vastly increasing the resolution and practical magnification. Transmission electron microscopy (TEM) works in a similar fashion to conventional light microscopy, producing an image devoid of depth information. Scanning electron microscopy works in a similar way to CLSM, by scanning a focused electron beam across the sample and detecting reflections, producing a two dimensional image with shadowing indicating depth in the sample.

Electron microscopy is unfortunately limited by a requirement of the electron beam to be housed in a vacuum, the delicate nature of biological specimens, and the fact that electron beams are not easily deflected. Consequently, specimens have to be thoroughly dried and coated with electron dense materials such as gold before they can be viewed, this treatment leads to the loss of biological features such as extracellular polysaccharide, and the generation of artifacts. To some extent these limitations are reduced in environmental scanning electron microscopes, in which

the sample chamber is separated from the main vacuum system. This allows higher pressures to be used in the sample chamber, permitting the examination of hydrated specimens.

1.5.2 Cultural

Overview

Microbiological analysis is strongly rooted in culture-based techniques, in particular the isolation of pure cultures to allow examination of individual taxa. Although molecular-based methods are becoming more and more powerful, culture-based analyses remain important and a pure culture is still required before a new taxon will be given a valid taxonomic name (Christensen *et al.*, 2001). The most common culture-based measure is the total viable count, which is usually obtained by performing a serial dilution of a bacterial suspension followed by plating out the suspension onto agar growth media. By manipulation of the growth conditions such as atmospheric gases and incorporation of inhibitory compounds in the media, viable counts can be obtained for specific groups of microorganisms.

Limitations

There are several serious problems with culture-based methods which have encouraged the development of molecular, microscopic, or other alternatives. In particular it is widely recognised that most microorganisms are culture-resistant, that is that they will not grow in the laboratory, or they will only do so in exceptional circumstances. Sometimes these bacteria are described as 'uncultivable' or similar, but this term suggests that the establishment of laboratory cultures is impossible so it is best avoided.

The oral microbiota of humans is a special case in respect of the proportion of species which have been cultured; due to the long history of experimentation, approximately 50% of the human oral microbiota has been cultured (Paster *et al.*, 2001), much more than the cultured fraction of bacteria from other ecosystems (e.g. 5% in soil; Bakken, 1985)

Another problem with viable counting is that many bacteria stick together by

virtue of their preferred biofilm mode of growth or as a consequence of the mechanics of cell division; any clumping of cells in the dilution series clearly results in a reduction in the apparent number of viable cells in the sample. For this reason viable counts are usually expressed in terms of colony forming units (cfu).

1.5.3 Molecular

Overview

Molecular analysis techniques are those which depend upon interactions and properties of nucleic acids, in particular their informational property. Each bacterial genome contains the instructions it needs to survive and replicate, and its transcriptome at any given time would give a snapshot of the genes presently being expressed. Given this wealth of information, it is no wonder that molecular biology has become such a powerful force in biology, however accessing and interpreting molecular data can be far from easy.

Genome sequencing is a big and expensive undertaking, and although the number of completed bacterial genomes is increasing week by week, the majority of the microbial world is unlikely to be sequenced for a long time. The power of environmental DNA sequencing, or metagenomics, has recently been spectacularly demonstrated by the sequencing of DNA extracted from the Sargasso Sea (Venter *et al.*, 2004); this supposedly simple ecosystem yielded a great diversity of genes and organisms, many of them new to science and together almost doubling the amount of sequence data on GenBank. Such extravagant projects are becoming more commonplace, but the required sequencing capacity, not to mention the downstream analysis remains for now out of the reach of most laboratories.

Comparison of community analysis methods

Many molecular tools rely on the polymerase chain reaction (PCR), which can be used to amplify specific DNA fragments in a mixture if the flanking sequences are known. The size of the amplicon can be determined on an agarose gel to confirm that the correct sequence was amplified, and it can then be subjected to DNA sequencing if further information is required. PCR is a general method which forms

part of many other protocols requiring DNA amplification, but on it's own PCR can be used to quickly detect specific DNA species within a mixed sample.

Practical molecular methods for analysis of microbial communities include PCR-cloning and sequencing, community profiling by denaturing gradient gel electrophoresis (DGGE), and detection of specific nucleotide sequences using microarray techniques. Any of these methods can be used to examine any nucleic acid target but they are often used in oral biology to give a phylogenetic snapshot or profile of the community based on 16S rRNA gene sequences.

The 16S rRNA gene is universally conserved among all cellular organisms due to its critical role in the ribosome during translation of genes to proteins. A peculiarity of this gene is that it is not itself translated to a protein, but performs its function as a single stranded structural RNA complexed with the ribosomal proteins. The structure of the molecule is largely determined by complementary base pairing between bases on the same strand, resulting in the formation of hairpin loops with double and single-stranded regions. This combination of properties fortuitously results in a gene with very highly conserved regions closely associated with variable and hypervariable regions. Thus, primers and probes can be designed with specificity for species, genus, or phylum. In addition universal primers can be used to amplify and sequence the whole gene economically, allowing phylogenetic comparisons to be easily made.

Each molecular method has it's own particular benefits and problems which will influence the choice of method used. Cloning community DNA or previously amplified genes (PCR cloning) has the advantage of generating libraries of large DNA fragments which can be probed for function or used for sequencing, but comparison between samples is difficult because of labour and sampling issues. DGGE can be used to fingerprint and compare samples, but is limited to comparison of relatively short DNA fragments. Microarrays potentially allow rapid throughput and discrimination of nucleic acids by hybridisation, but detection limit problems and a general requirement of prior knowledge about the target sequences, combined with the technical challenge and cost of making arrays, limit their use.

1.5.4 Metabolic

Metabolic tests may be performed on pure cultures or on mixed cultures, and can reveal information about the phenotype of the organism or community being investigated. Typical metabolic tests include the oxidase and catalase tests, which can quickly show whether the sample has cytochrome oxidase activity or the capacity to break down hydrogen peroxide respectively. Bacterial taxonomy has been until recently largely based upon such phenotypic tests, which may now seem old fashioned with the advent of molecular taxonomy. Such tests, however, still have an important place in microbiology because they define an organism or community by what it is, by observable function, rather than by potential implied by the genome.

Metabolic profiling of soil communities has been shown to be practical and a commercial kit is available specifically tailored to this (Garland and Mills, 1991). Such analyses could also be very useful for studying oral communities, especially for rapid metabolic profiling of microcosms generated by laboratory models. This could be particularly powerful when combined with molecular profiling techniques such as DGGE, as it may be possible to correlate changes in community structure to changes in metabolic potential and use this as a means to ascribe function to groups of organisms resistant to axenic culture.

1.6 Aims and objectives

From extensive research in humans, it has become clear that an understanding of the microbial ecology of the mouth is fundamental to elucidating the aetiology of most oral diseases (Schenkein, 1999), yet the oral ecology and microbiology of the dog remain largely uncharacterised.

The aim of this work was to adjust the oral microbiota literature bias in favour of the dog; by characterising the canine oral microbiota, examining some of it's representatives, and developing a laboratory model of canine dental plaque to empower further research. In addition, it is hoped that this work will have a broader relevance, particularly in the fields of oral microbiology and microbial ecology, and the ultimate aim is that this work will support future development of oral healthcare for dogs.

Chapter 2

Materials and methods

This chapter details general methods and information which were used throughout this study. Specific methods which relate only to a particular chapter are explained in the methods section of the relevant chapter.

2.1 Cohorts

All plaque samples were taken from dogs undergoing routine dental treatment, either at the Waltham Centre for Pet Nutrition (Waltham-on-the-Wolds, UK), or at Beech House veterinary surgery (Surrey, UK). Saliva samples were taken from dogs housed at the Waltham Centre for Pet Nutrition. Dogs housed at the Waltham Centre for Pet Nutrition were kept in environmentally enriched facilities, and dogs sampled at Beech House were companion animals.

The dogs sampled were four Miniature Schnauzers (female neutered), three Cocker Spaniels (male; 1 entire, 2 neutered), one Cairn Terrier (female), and one Collie cross (female neutered). The Cairn Terrier had Cushings syndrome and was taking trilostane (Arnolds Veterinary Products Ltd, UK), a steroid synthesis inhibitor. None of the dogs had received antibiotics in the three months prior to sampling.

2.2 Clinical scoring

Clinical scoring was performed after sampling. The parameters measured were periodontal probing depth (PPD), bleeding on probing (BOP), and the gingival index (modified for veterinary dentistry from Löe, 1967) as set out in Table 2.1.

Score	Criteria
0	no gingivitis
1	Slight inflammation, i.e. slight redness but no bleeding on probing.
2	Mild inflammation, i.e. slight redness and swelling, with delayed bleeding on gentle probing of the gingival sulcus.
3	Moderate inflammation, i.e. the gingiva is red, swollen, and bleeds on gentle probing of the sulcus.
4	Severe inflammation, i.e. the gingiva is red or reddish-blue, the gingival margin is swollen, tendency to spontaneous haemorrhage on probing and/or ulceration along the gingival margin.

Table 2.1: Gingival index, modified for veterinary dentistry from Löe 1967.

2.3 Sampling

2.3.1 Saliva

Canine saliva was collected in two ways; a ball was used to make the dog salivate, and then the saliva was syringed out of the mouth, or salivettes were used. After collection, 100% dextran was added to each sample at a ratio of 25% v/v; samples from 5 dogs were pooled and stored at -70°C. After approximately 50 ml had been collected, the pooled saliva was thawed and mixed, and 1 ml aliquots were dispensed into cryovials stored at -70°C to provide a standard inoculum for future work.

2.3.2 Plaque

Plaque sampling was performed under general anaesthesia prior to clinical scoring by a qualified person. Dogs were premedicated with acepromazine and buprenophine

by subcutaneous injection. Anaesthesia was induced with Propofol and maintained on isoflurane with oxygen via a cuffed endotracheal tube.

Individual plaque samples were taken with a curette or dental probe from the gingival margin, except where a periodontal pocket was present, in which case the sample was taken from the base of the pocket. Nine plaque samples were taken from the buccal surfaces of upper second or third premolars of the nine dogs, based upon established common practice (e.g. Syed *et al.*, 1981) and the observations of Sorensen *et al.* (1980), who found the buccal surfaces to harbour the most plaque, increasing from the canines to a maximum on the maxillary 4th premolar, and then decreasing in the molar regions. In addition, a single supragingival plaque sample was taken from an upper second premolar, and a distal/mesial pooled sample was taken from the periodontal pocket on an upper second incisor (202), both of these extra samples being taken from one of the nine dogs mentioned previously. Samples were immediately transferred to a vial containing 1 ml of reduced transport fluid (Syed and Loesche, 1972) for transportation to the laboratory.

A pooled plaque sample (CPP01) was taken from the entire dentition of three miniature schnauzers (Petal, Poppy, Daisy) using sterile swabs to remove supragingival plaque, reaching as close to the gingival margin as possible. The swabs were placed immediately into a universal bottle containing 20 ml reduced transport fluid (RTF) and transported to the laboratory. The sample was vortex-mixed for two minutes, the suspension was transferred to a centrifuge tube, and then centrifuged for 15 minutes at 3000 RCF. The supernatant was removed and replaced with BHI and 10 % glycerol, before being vortex-mixed and dispensed in 1 ml aliquots into cryovials and stored at -70°C. Details of all plaque samples are summarised in Table 2.2.

2.4 Suppliers of chemicals and reagents

Unless stated otherwise, chemicals were supplied by Sigma (Poole, UK) or BDH (Poole, UK), growth media were supplied by Oxoid (Basingstoke, UK), and PCR primers were supplied by Sigma Genosys (Cambridge, UK).

Sample ID	Location	GI	PPD	Dog	Breed	Age
cp01◇	206	nd	nd	Rosie	MS	6y7m
cp02	106	nd	nd	Rosie	MS	6y7m
cp03	106 α	nd	nd	Rosie	MS	6y7m
cp04	207	1	0	Stumpy	CS	1y9m
cp05	107	2	0	Errol	CS	1y9m
cp06	107	2	0	Midas	CS	2y3m
cp07	107	3	2	Petal	MS	7y11m
cp08	107	0	2	Poppy	MS	7y11m
cp09	107	1	2	Daisy	MS	7y11m
cp10	202 β	2	5	Daisy	MS	7y11m
cp17	207	0	5	Susie	CT	11y1m
cp62	107	2	4	Dica	CC	13y2m
cp11–cp33	left γ	0-3*	0-5*	Susie	CT	11y1m
cp34–cp62	entire δ	0-4*	0-5*	Dica	CC	13y2m

Table 2.2: Summary of plaque samples showing the gingival index (GI) and periodontal probing depth (PPD) for each sampled tooth.
α supragingival, β distal and mesial, γ entire left dentition, δ entire dentition, * further information given in Table 4.5, ◇ tooth surface cleaned with chlorhexidine before sampling.
Breed abbreviations; MS Miniature Schnauzer, CS Cocker Spaniel, CT Cairn Terrier, CC Collie cross medium sized.

2.5 Growth Media

2.5.1 Agars

The growth media used for making viable counts were anaerobe agar (Bioconnections, Leeds, UK) with 5% defibrinated horse blood (E&O Laboratories, Bonnybridge, UK) (AA) for total anaerobes, Columbia agar base (Oxoid) with 5% defibrinated horse blood (CBA) for total aerobes, *Veillonella* agar (Difco, West Molesey) for *Veillonella* species, Mitis Salivarius agar (MS, Difco) for streptococci, and triple strength cadmium fluoride acriflavin tellurite agar (CFAT) (Zylber and Jordan, 1982) for *Actinomyces* species.

Plates were incubated aerobically with 5 % CO_2, or in an anaerobic chamber (MACS 1000, Don Whitley Scientific, Shipley, Yorkshire, UK) with an atmosphere containing 80 % nitrogen, 10 % hydrogen, and 10 % carbon dioxide at 37°C. After

3 days (aerobic plates only), and again after 7-10 days, the plates were examined and each morphotype was counted and subcultured to obtain pure cultures.

2.5.2 Canine artificial saliva

The growth medium fed to the CDFF and used in other experiments was a modification of the complete artificial saliva (AS) described by Pratten *et al.* (1998), termed canine artificial saliva (CAS) in this study. Pratten *et al.* (1998) developed their complete saliva based upon the artificial salivas previously formulated by Russell and Coulter (1975) and Shellis (1978); they found that combining elements of each and increasing the amount of mucin resulted in higher growth rates and final cell densities in their *S. sanguis* biofilms. Later work has also showed that this formulation is well suited for generation of microcosm plaque biofilms (e.g. Pratten and Wilson (1999)), although microcosm studies using AS to date have mostly been supragingival plaque models inoculated with human saliva.

CAS used in this study contains different salt concentrations compared to AS and is adjusted to pH 7.5, based upon measurements of the salts content and pH of canine saliva made at the Waltham Centre for Pet Nutrition, and on the observations of Harvey *et al.* (1995). In addition, horse serum (Oxoid) was added at a rate of 5 % v/v to some microcosm experiments to simulate gingival crevicular fluid. Table 2.3 shows the ingredients used to make CAS.

Ingredient	quantity per litre
Lab-lemco powder (Oxoid)	1.0 g
Yeast extract (Oxoid)	2.0 g
Proteose peptone (Oxoid)	5.0 g
Hog gastric mucin (Sigma)	2.5 g
Sodium chloride	2.3 g
Calcium chloride	0.1 g
Potassium chloride	1.5 g
40% Urea	1.25 ml

Table 2.3: Composition of Canine Artificial Saliva. Before autoclaving, the pH of the medium was adjusted to 7.5 using 5 M sodium hydroxide. Urea was filter-sterilised and added after autoclaving. All salts are anhydrous masses.

2.6 Microscopy

2.6.1 Light microscopy

Light microscopy was carried out with brightfield illumination typically using a 32× objective or 100× oil immersion objective. Wet preparations were covered with a cover slip.

2.6.2 Scanning electron microscopy

Biofilms for SEM were placed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer and stored at 4°C for at least 24 hours to fix the samples. Fixed biofilms were dehydrated in an alcohol series by gently applying from 20 % to 100 % ethanol, finishing with three changes of 100 % ethanol for 10 minutes each. The dehydrated biofilms were then covered with hexamethyldisilazane, then removed after 1-5 minutes and placed in a dessicator overnight to dry. Dried specimens were mounted onto aluminium stereoscan stubs and coated with a thin metallic layer of gold/palladium in a Polaron E5000 sputter coater (BioRad, Hercules, CA, USA). The prepared biofilms were visualised using a Cambridge 90B SEM and images were captured digitally.

2.6.3 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to visualise bacterial cell surfaces and coaggregates, using methylamine tungstate as a negative stain. Negative staining of cells was performed on plasma glowed formvar / carbon coated 400 mesh copper grids (Agar Scientific, Essex). The following protocol, based on the method of Handley *et al.* (1985), was performed quickly to prevent the grid from drying:

1 Float 20 μl culture 1 on top of grid for 1 minute.

2 Draw off culture with a damp filter paper.

3 Float 20 μl culture 2 on top of grid for 1 minute (if required), and draw off.

4 Float 20 μl 1 % w/v methylamine tungstate on top of the grid for 1 minute and draw off.

2.6.4 Confocal laser scanning microscopy

Substrata with attached hydrated biofilms from the CDFF were secured into the base of small petri dishes using silicone grease (Dow Corning, Midland, MI, USA) and covered with physiological saline containing fluorescent stains. The stains used were either Syto13 (Molecular Probes, Eugene, Oregon, USA), or BacLight™ bacterial viability kit (Molecular Probes). Syto13 is a general DNA stain used to visualise all of the cells in a biofilm (fluorescence is much greater in the bound state), and BacLight™ is a two part stain which differentially stains live and dead cells based upon membrane integrity and metabolic activity. Syto13 applied at 0.03 % v/v, and BacLight™ was prepared by adding 10 μl of each kit component to 10 ml saline. Images were obtained using a Radiance 3000 confocal laser-scan head (Bio-Rad GmbH, Jena, Germany), in conjunction with a BX51 stereomicroscope (Olympus UK Ltd, Southall, UK), and a 40× HCX water immersion dipping lens. Image processing was performed using ImageJ (Rasband, 2005).

2.7 DNA extraction

DNA was extracted from samples using a commercial DNA extraction kit (Puregene Gentra Systems, Minneapolis, MN, USA). The manufacturers protocol for Gram-positive bacteria was followed, except that centrifugation times for pelleting cells and DNA pellets were increased to five minutes.

2.8 DNA purification

PCR products were cleaned using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) to remove excess nucleotides, enzymes, genomic DNA, and other PCR mastermix components. Cleaned DNA was typically eluted to a volume of 30 μl.

2.9 Polymerase chain reaction

For amplification of DNA from pure bacterial cultures on agar, a small amount of a single colony was picked with a sterile cocktail stick and mixed into 50 μl water in a PCR tube. PCR mastermix was then added and the initial PCR denaturation was usually sufficient to release DNA from the cells. For biofilm samples or recalcitrant pure cultures, DNA extraction was performed and an aliquot was used for the PCR template.

2.10 DNA fragment analysis

PCR products were checked on a 1 % agarose gel with 0.1 μl ml^{-1} ethidium bromide. Gels were loaded with 1 μl sucrose loading buffer and 5 μl PCR reaction, and run for 50 Vh, before being photographed using a gel imaging system with digital camera (Alphaimager, Alpha Immotech, San Leonardo, CA, USA). PCR DNA marker (Amresco, Luton, UK) containing markers at 50, 150, 300, 500, 750, 1000, 1500, and 2000 bp positions was loaded onto gels to assist estimation of DNA fragment lengths.

2.10.1 Primers

Most PCR primers used in this study were targeted at the 16S rRNA gene. Table 2.4 shows all of the universal primers used for general amplification of this gene. Primers used for DGGE, and primers targeted at specific phylogenetic groups are listed in Section 4.2.2 and Section 4.2.3 respectively.

2.10.2 Standard PCR conditions

Most PCR reactions were performed in 100 μl volumes using the mixture shown in Table 2.6. The standard cycling parameters were as shown in Table 2.5.

Name	Sequence (5'-3')	Reference
27F	AGAGTTTGATCMTGGCTCAG	Lane (1969)
338F	AACTGAGACACGGTCCAGAC	Lane (1969)
357F	CTCCTACGGGAGGCAGCAG	Lane (1969)
519R	GTATTACCGCGGCTGCTG	Lane (1969)
907R	CCGTCAATTCMTTTRAGTTT	Lane (1969)
926F	AAACTYAAAKGAATTGACGG	Lane (1969)
1100R	GGGTTGCGCTCGTTG	Lane (1969)
1114F	GCAACGAGCGCAACCC	Lane (1969)
1492R	TACGGYTACCTTGTTACGACTT	Lane (1969)
M13F (-20)	GTAAAACGACGGCCAG	Invitrogen protocol*
M13R	CAGGAAACAGCTATGAC	Invitrogen protocol*

Table 2.4: Universal PCR primers for amplification of bacterial 16S rRNA gene. F=forward, R=reverse, numbers indicate gene position using *E. coli* numbering (except M13 primers). M=C:A, Y=C:T, K=G:T, R=A:G; all 1:1.
* see Section 2.13.

Lid	Repeat	Condition
110°C		94°C 5 min
	× 29	94°C 1 min
		54°C 1 min
		72°C 1.5 min
		72°C 5 min
off	pause	4°C

Table 2.5: Standard PCR cycle.

2.11 DNA sequencing

DNA sequencing was carried out using the chain terminator method of Sanger *et al.* (1977) on two different machines. Initial sequencing was done on an ABI 310 Prism Genetic analyser (Applied Biosystems, Warrington, UK), and later sequencing to increase coverage of existing reads was done on a Beckman Coulter CEQ2000 automated DNA sequencer (Beckman Coulter, High Wycombe, UK). Output from the machines was in the form of electropherograms, which were analysed with Chromas v1.43 (McCarthy, 1997) to obtain the DNA sequence data.

Ingredient	supplier	volume (μl)
sterile deionised H_2O		76.7
10x (Biotaq)	Bioline	10.0
dNTPs (10 mM)	Promega	2.0
$MgCl_2$ (Biotaq)	Bioline	5.0
Forward primer (10 μM)	Sigma-Genosys	3.0
Reverse primer (10 μM)	Sigma-Genosys	3.0
Taq (Biotaq)	Bioline	0.3
Total		100.0

Table 2.6: PCR mastermix for one reaction. Template volume was typically 1 μl, and the H_2O volume was adjusted accordingly.
Suppliers: Bioline, London, UK; Promega Life Science, Southampton, UK; Sigma-Genosys, Cambridge, UK.

Ingredient	ABI	Beckman
Cleaned DNA template	1 μl	1 μl
dH_2O	3.5 μl	-
primer (10 μM)	0.5 μl	1 μl
Bigdye	2 μl	-
Quickstart	-	8 μl

Table 2.7: Sequence PCR mastermixes for one reaction, for ABI 310 and for Beckman CEQ2000.

Sequence PCR protocol (ABI)

Sequencing PCR reactions were prepared as shown in Table 2.7, and run on the program shown in Table 2.8.

Lid	Repeat	Condition
105°C	× 99	95°C, 10 s
		50°C 5 s
		60°C 4 min
off	pause	4°C

Table 2.8: Sequence PCR thermal cycling parameters for ABI 310.

Sequences were cleaned and analysed as follows. To each reaction, 15 μl water, 2 μl 3M sodium acetate and 50 μl 95% ethanol (-20°C) were added before incubation on ice for 20 minutes. The precipitated reactions were then centrifuged at

14,000 RCF for 25 minutes at 4°C. The supernatant was removed by pipette, and 250 μl 70% ethanol (-20°C) was added before being centrifuged again at 14,000 RCF for 15 minutes at 4°C. The supernatant was removed and tubes were then dried at 95°C for a few seconds with the lid removed. The extract was re-suspended in 20 μl TSR (Template Suppressor Reaction, Applied Biosystems) and vortexed, then heated at 95°C for 2 minutes, vortexed again and then placed on the sequencer for analysis.

Sequence PCR protocol (Beckman)

Sequencing PCR reactions were prepared as shown in Table 2.7, and run on the thermal cycling program shown in Table 2.9.

Lid	Repeat	Condition
105°C	× 30	96°C 20 s
		50°C 20 s
		60°C 4 min
off	pause	4°C

Table 2.9: Sequence PCR thermal cycling parameters for Beckman CEQ2000.

Sequences were cleaned up and analysed according to the manufacturer's instructions in 96 well PCR plates (Beckman Coulter).

2.12 DNA sequence assembly and comparison

When multiple sequence reads were obtained for a single template they were assembled using BioEdit (Hall, 1999) and contig assembly program (Huang, 1992).

To obtain identifications based upon 16S rRNA gene sequences, comparisons were made to sequences available in public databases using the provided online tools. BLAST (Altschul *et al.*, 1990) searches were done on sequences in the GenBank database (Benson *et al.*, 2004), and the sequence match tool was used on sequences held on the ribosomal database project (Cole *et al.*, 2003). Each of these tools returns a list of matches ranked by a probability score. Sequence alignment

and further comparisons were made in many cases to allow building of phylogenetic trees, and this is described in Section 3.2.4.

2.13 PCR-Cloning

DNA amplified by PCR from a mixed template of community DNA is likely to include a mixture of species which cannot be sequenced directly. Individual amplicons from such mixtures were cloned into *E. coli* using a TOPO TA Cloning® Kit for Sequencing (Invitrogen, San Diego, CA, USA), via a plasmid vector according to the manufacturers instructions. Cloned DNA was amplified by PCR using the M13 forward and reverse primers flanking the insertion site, and the resulting products were used as a template for DNA sequencing.

Chapter 3

Culture-based analyses

3.1 Introduction

The canine oral microbiota was analysed by obtaining pure culture isolates from plaque and saliva, and identifying them by comparative 16S rRNA gene sequencing. Although many previous workers have performed similar examinations, the vast majority have relied upon conventional identification methods and had a bias towards understanding of the human microbiota. In particular, the dog has often been used as an animal model for human oral diseases such as periodontal diseases (Weinberg and Bral, 1999; Madden and Caton, 1994), and it has also received attention because of the bacteria it may transfer to people, for example through biting (Allaker *et al.*, 1997a; Forsblom *et al.*, 2002). In this respect, it has been recognised that the dog has a distinct oral microbiota as it is widely believed that bites from dogs are less dangerous in terms of wound infection potential compared to human bites (Goldstein, 1992).

The large research effort directed at the human oral microbiota provides an excellent data set for comparison with results from the present study. Such comparisons are used to consider the evolution of the bacterial communities involved, their respective niches, and the possibility of different species filling similar niches in different hosts. If such species can be identified, they may provide useful information regarding human and animal oral diseases by providing a basis for uncovering universal characteristics of pathogenic and protective species.

3.2 Materials and methods

3.2.1 Samples

Canine plaque and saliva samples were collected as described in Section 2.3.

3.2.2 Viable counting

Samples were serially diluted in PBS, and aliquots were plated out onto CBA and AA. Plates were incubated aerobically with 5 % CO_2 or in an anaerobic cabinet (MACS 1000, Don Whitley Scientific, Shipley, Yorkshire, UK) as appropriate at 37°C. After 3 days (aerobic only), and after 7-10 days the plates were examined and each morphotype was counted and subcultured to obtain pure cultures.

3.2.3 Isolate identification and storage

Pure isolates obtained from viable count plates were characterised by Gram-stain, catalase test, and oxidase test. Identifications were made by comparing partial 16 S rRNA gene sequences as described in Section 3.2.4, and the above phenotypic tests were used to support these identifications.

Catalase

Isolates were tested for catalase by transferring a small amount of the colony to a glass slide, then adding a drop of 0.02 % hydrogen peroxide, and a positive result was recorded if effervescence occurred.

Oxidase

Isolates were identified as oxidase-positive if within about 10 seconds, contact with a swab moistened with NNN'N'-Tetramethyl-p-phenylenediaminedihydrochloride (BDH) in water produced a purple colour.

Gram-stain

A light smear of cells was gently heat fixed to a glass slide. Crystal violet was added for 30 seconds, then chased away with iodine, which was left in place for 30 seconds. The slide was rinsed with water and decolourised briefly with acetone before being counterstained with safranin for one minute and rinsed with water. Slides were gently blotted and allowed to air dry before viewing with a 100× oil immersion objective.

Isolate storage

All isolates were preserved by mixing a large amount of pure strain (e.g. a whole plate) into a sterile 1:1 mixture of BHI and glycerol using a sterile cotton swab, and stored in a cryovial at -70°C.

3.2.4 Comparative DNA sequence analyses

Partial 16S rRNA gene sequences were obtained from pure cultures as described in Section 2.11.

Sequence alignment

Sequences were aligned using ClustalX (Thompson *et al.*, 1997), and all gaps were removed using BioEdit (Hall, 1999). For short alignments of less than 400 bases, some gaps were sometimes allowed to remain in order to make maximum use of the available data.

Distance matrices and percent identity calculations

Distance matrices were calculated from de-gapped alignments using the DNAdist program of the Phylip software package (Felsenstein, 1993). Distance matrices for generation of phylogenetic trees were corrected for multiple substitutions using the method of Jukes and Cantor (1969). To calculate percentage identity between pairs of sequences, degapped alignments were compared using BioEdit without using algorithms for correction of multiple substitutions.

Phylogenetic trees

Neighbour-joining phylogenetic trees were produced from distance matrices using the Neighbour program of the Phylip software package (Felsenstein, 1993). All phylogenetic trees are unrooted unless specified otherwise. The sequences from the best matches on BLAST (Altschul *et al.*, 1990) and RDP (Cole *et al.*, 2003) for each isolate were included on the trees to provide a visual representation of the relatedness of the isolated bacteria to named bacteria from GenBank. All sequences originating from this study are displayed in green typeface.

3.2.5 Phylogenetic analyses

Initial identification and phylotype assignment

All isolates were initially identified by performing database searches on the 16S rRNA gene sequences obtained from a single read of approximately 300-400 bases, targeting the hypervariable V3 region of the gene using the 357F primer as described in Section 2.11. Sequences were then put into groups (typically 1-3 genera) to produce phylogenetic trees of related bacteria. These trees revealed natural groupings between the isolated bacteria which were used to arrange them into groups termed phylotypes, since exact species or genus information were not known at his stage. Phylotypes were assigned according to database search results and the distance separating known related species on the same tree, so that each phylotype should approximately represent a distinct species.

Phylotype identification

Once all of the isolated bacteria had been assigned to a phylotype, a second round of sequencing was performed as described in Section 2.11 to obtain more accurate sequence data for representatives of each phylotype. These data are termed high fidelity sequences, and are defined by having a minimum of double coverage for at least 600 bases. Due to time constraints, this standard was not achieved for all phylotypes isolated.

High fidelity sequences for each phylotype were subjected to further database searching and tree analyses to obtain accurate phylogenetic data for the originating

organism. Database searches, phylogenetic trees, and percent sequence homology to database sequences were used to identify each phylotype to species level where possible.

A difference of 3 % or greater was taken to indicate that sequences were probably from distinct species (Goebel and Stackebrandt, 1994; Forney *et al.*, 2004). Sometimes more similar sequences were considered distinct species if other described species of the genus were particularly homogeneous. The above methods were chosen and applied in order to maximise the number of identifiable species from the sequence data, and therefore minimise the possibility of erroneously flagging a sequence as originating from a previously undescribed species.

These identifications are assumed to be indicative of the identity for each phylotype as a whole since initial sequencing data showed their affinity.

3.2.6 Literature search

To check if isolated bacterial species were part of the indigenous human oral microbiota, the following search was performed on pubmed[1] using the current valid name and all previous and invalid names:

genus AND species AND (plaque OR saliva OR oral)

Database hits were followed up to determine whether the bacterium had been detected from a human mouth, and if so it was considered to be a member of the indigenous oral microbiota.

3.3 Results

3.3.1 Phylogenetic diversity

A total of 339 bacterial isolates were recovered by culture from the dental plaque of seven dogs and a pooled saliva sample. From these isolates, 84 different bacterial phylotypes belonging to 37 genera were identified by 16S rRNA gene sequencing,

[1] 12 October 2004

and 34 isolates remained unidentified. Phenotypic data and sequence related results for all isolates are shown in Appendix A.

In four cases, multiple phylotypes were identified as the same species, but the original phylotype designations were retained because these may represent distinct strains. An average of 17 distinct phylotypes were detected from each plaque sample, and 22 phylotypes were detected from the pooled saliva sample. Sequences used to identify phylotypes have been deposited in GenBank with accession numbers AY827856 - AY827945.

Figure 3.1 shows an example phylogenetic tree as used to group the isolated bacteria for each group of related taxa.

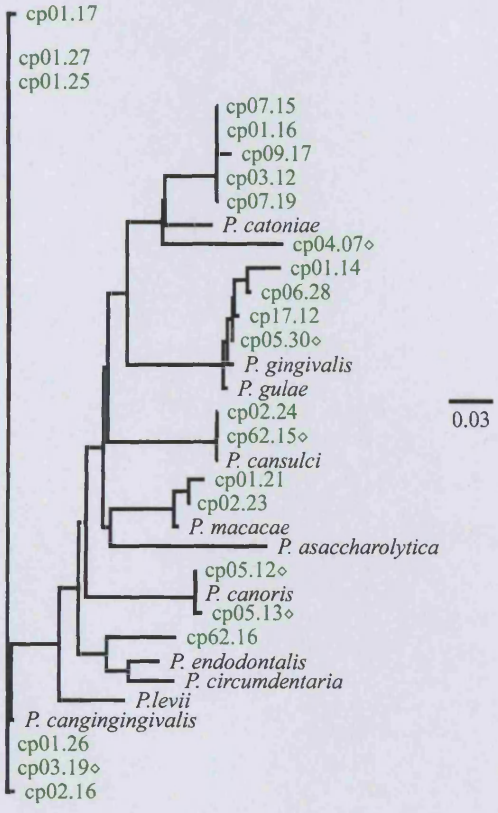


Figure 3.1: Neighbour joining tree showing phylotype designations for *Porphyromonas* species, using 201 alignment positions. High fidelity sequences were later obtained for the highlighted isolates (◇). Scale bar indicates 3 % sequence divergence.

Longer sequences were used to further characterise some groups by database searches and additional phylogenetic trees as shown in Figure 3.2. This figure shows the highlighted isolates from Figure 3.1 using high fidelity sequences. Similar analyses were performed for all of the high fidelity sequences.

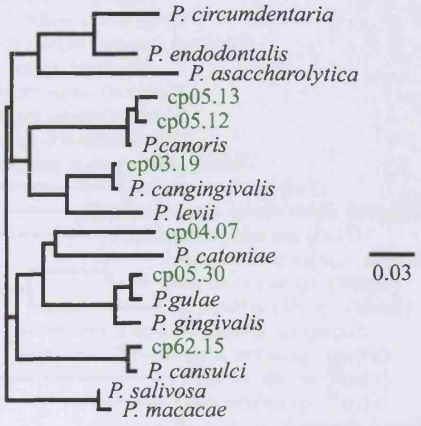


Figure 3.2: Neighbour joining tree showing high fidelity sequences from *Porphyromonas* species, along with database sequences for the closest BLAST matches and selected *Porphyromonas* species (491 alignment positions). Scale bar indicates 3 % sequence divergence.

Phylotype identifications were combined with viable counting results from primary isolations to determine the frequency at which each group was isolated for each sample category (Section 3.2.2). A summary tree showing all of the phylotypes for which high fidelity sequences were obtained is shown in Figure 3.3.

Of the 84 phylotypes, species level identifications were obtained for 40 phylotypes as summarised in Table 3.1, the remainder could not be reliably identified to species level and these are summarised in Table 3.2. Only 28 % of species identifications are considered indigenous human oral microbes based on a literature search.

For 44 phylotypes (52 % of total) there was no similar sequence available on GenBank to provide a species identification. These are tentatively identified as probable new species or genera, especially where it can be determined that GenBank does in fact hold a sequence for all valid members of the genus (Table 3.2).

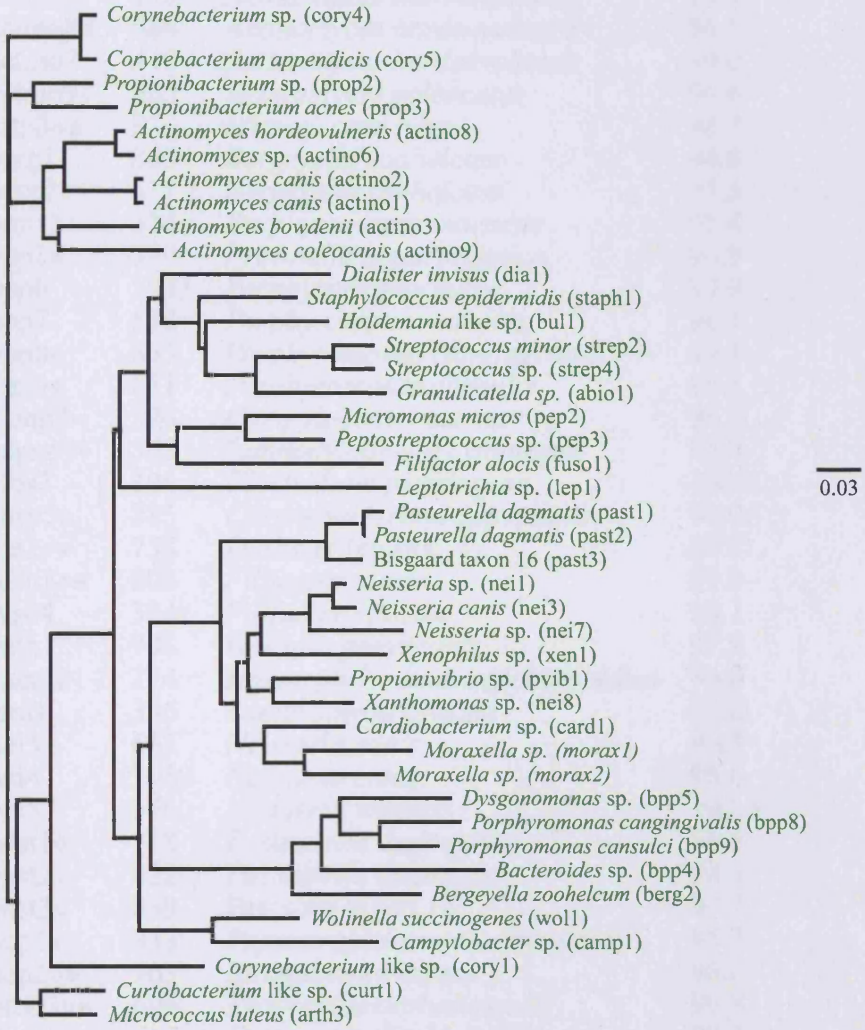


Figure 3.3: Neighbour joining tree showing all phylotypes for which high fidelity sequences were obtained. Sequences are identified by their originating species or genus identification with the phylotype in brackets. This tree is based on an alignment of 371 positions, significantly less than the length of the original sequences because the sequences of diverse organisms introduce gaps at different positions in the alignment. Scale bar indicates 3 % sequence divergence.

ptype	bp	species	% ID
actino1◇	643	*Actinomyces canis*	96.7
actino2◇	697	*Actinomyces canis*	99.4
actino3◇	697	*Actinomyces bowdenii*	99.7
actino5	396	*Actinomyces hordeovulneris*	98.4
actino8◇	684	*Actinomyces hordeovulneris*	96.1
actino7	440	*Actinomyces hordeovulneris*	99.0
actino9◇	605	*Actinomyces coleocanis*	96.6
arth3◇•	814	*Micrococcus luteus*	98.7
berg1	868	*Bergeyella zoohelcum*	94.8
berg2◇	611	*Bergeyella zoohelcum*	95.5
bpp11	334	*Porphyromonas macacae*	95.4
bpp3•	399	*Prevotella heparinolytica*	96.9
bpp6	1083	*Porphyromonas gulae*	99.9
bpp7	538	*Porphyromonas canoris*	96.7
bpp8◇	655	*Porphyromonas cangingivalis*	99.1
bpp9◇	611	*Porphyromonas cansulci*	98.5
camp2•	374	*Campylobacter curvus*	96.7
capno1◇	362	*Capnocytophaga cynodegmi*	97.4
clos2	304	*Clostridium perfringens*	100.0
cory5◇	885	*Corynebacterium appendicis*	98.0
dia1◇•	758	*Dialister invisus*	99.8
fuso1◇•	808	*Filifactor alocis*	99.0
fuso4	384	*Filifactor villosus*	98.1
gem1	908	*Gemella palaticanis*	97.9
haem2	274	*Haemophilus haemoglobinophilus*	96.6
lam1	336	*Lampropedia hyalina*	97.0
nei3◇	663	*Neisseria canis*	99.5
nei4	1089	*Neisseria canis*	98.6
nei5	346	*Neisseria weaveri*	100.0
past1◇	718	*Pasteurella dagmatis*	98.5
past2◇	822	*Pasteurella dagmatis*	98.3
past3◇	839	Bisgaard taxon 16	97.7
pep1•	333	*Peptostreptococcus anaerobius*	98.7
pep2◇•	703	*Micromonas micros*	96.0
prop3◇•	696	*Propionibacterium acnes*	99.8
rhod1•	417	*Dietzia psychralcaliphila*	98.7
staph1◇•	638	*Staphylococcus epidermidis*	99.6
strep2◇	634	*Streptococcus minor*	98.3
strep3•	403	*Streptococcus bovis*	99.2
woll◇•	857	*Wolinella succinogenes*	98.5

Table 3.1: Phylotypes identified to species level. ◇ indicates that high fidelity sequence was used for this phylotype identification. • indicates species considered to be members of the indigenous human oral microbiota. Detailed phylogenetic and phenotypic data for all isolates are given in Appendix A.

ptype	bp	Genus	BLAST	% ID
abio1◇	621	*Granulicatella*	*G. balaenopterae*	91.2
actino4Δ	358	*Actinomyces*	*A. slackii*	98.8
actino6◇	658	*Actinomyces*	*A. hordeovulneris*	94.9
actino10Δ	369	*Actinomyces*	*A. suimastitidis*	98.9
arth1	473	*Rothia*	*Rothia nasimurium*	92.7
arth2Δ	358	*Curtobacterium*	*C. flaccumfaciens*	95.2
bpp1	402	*Porphyromonas*	*P. catoniae*	90.1
bpp5◇	702	*Dysgonomonas*	*Bacteroides* sp.	92.4
bpp10	335	*Porphyromonas*	*P. endodontalis*	85.1
bpp12	902	*Stenotrophomonas*	*S. maltophilia*	91.8
bul1◇	655	*Holdemania*	*Solobacterium* sp.	84.6
bpp2	413	*Prevotella*	*P. ruminicola*	82.5
bpp4◇	833	*Bacteroides*	*B. uniformis*	91.5
camp1◇	625	*Campylobacter*	*C. rectus*	95.1
clos3	470	*Clostridium*	*C. leptum*	85.5
cory2	838	*Corynebacterium*	*C. falsenii*	95.4
capno2	1065	*Capnocytophaga*	*C.gingivalis*	89.4
card1◇	890	*Cardiobacterium*	*C.valvarum*	93.0
clos1	421	*Clostridium*	*C. hathewayi*	94.4
clos4Δ	175	*Clostridium*	*C. litorale*	93.1
cory1◇	683	*Corynebacterium*	*C. bovis*	79.6
cory3	851	*Corynebacterium*	*C. ciconiae*	93.1
cory4◇	737	*Corynebacterium*	*C. macginleyi*	94.1
cory6	227	*Corynebacterium*	*Corynebacterium* spp.	94.2
curt1◇	639	*Curtobacterium*	*Curtobacterium* sp.	94.0
fuso2	420	*Eubacterium*	*E. oxidoreducens*	85.0
Fuso3	1101	*Fusobacterium*	*F. nucleatum*	96.1
haem1	843	*Haemophilus*	*H. haemoglobinophilus*	93.3
haem3Δ	410	*Haemophilus*	*H. paraphrophilus*	95.5
lac1	580	*Streptococcus*	*S. infantarius*	90.4
lep1◇	852	*Leptotrichia*	*S. moniliformis*	85.6
morax1◇	728	*Moraxella*	*M. osloensis*	89.4
morax2◇	654	*Moraxella*	*M. cuniculi*	95.1
nei1◇	619	*Neisseria*	*N. dentiae*	96.0
nei7◇	892	*Neisseria*	*N. elongata*	94.4
nei8◇	876	*Xanthomonas*	*Xanthomonas* sp.	95.0
pep3◇	696	*Peptostreptococcus*	*Helcococcus* sp.	89.5

Table 3.2 – Continued on next page

Table 3.2 – Continued from previous page

ptype	bp	Genus	BLAST	% ID
prop1	592	*Tessaracoccus*	*T. bendigoniensis*	93.5
prop2◇	654	*Propionibacterium*	*F. spumicola*	91.7
pvib1◇	607	*Propionivibrio*	*P. dicarboxylicus*	93.2
strep1Δ	343	*Streptococcus*	several spp.	99.4
strep4◇	703	*Streptococcus*	*S. bovis*	91.8
xen1◇	572	*Xenophilus*	*X. azovorans*	94.8
xen2	855	*Xenophilus*	*X. azovorans*	92.9

Table 3.2: Phylotypes not represented on GenBank. The closest positively identified match from a BLAST search is shown, along with uncorrected percentage sequence homology between the two sequences. †indicates that sequences for all valid species of the genus were available for comparison (all subspecies were not necessarily available). ◇ indicates that high fidelity sequence was used for this phylotype identification. Δ indicates that there was insufficient information to identify a phylotype to species level or to identify it as novel. Detailed phylogenetic and phenotypic data for all isolates are given in Appendix A.

3.3.2 Viable Counts

Total viable counts from plaque samples were all between 1.9×10^3 and 6.4×10^6 cfu ml^{-1}, and the total viable count from the pooled saliva sample was approximately 1.0×10^6 cfu ml^{-1}. Figure 3.4 shows the genera isolated from plaque and saliva samples as a proportion of the total viable count for each sample. Overall, *Actinomyces* species were the most abundant, composing 11.6 % of the plaque microbiota and 25.5 % of the saliva microbiota. No other genera were represented at over 5 % in both plaque and saliva. *Granulicatella* and *Streptococcus* species were abundant in saliva at 16.5 % and 18.2 % respectively, whilst *Porphyromonas* and *Neisseria* species (along with *Actinomyces* spp.) dominated the plaque microbiota at 20.0 % and 10.3 %. All 37 identified genera except for *Staphylococcus* were found in plaque samples, but only 10 genera were found in saliva samples (27 %).

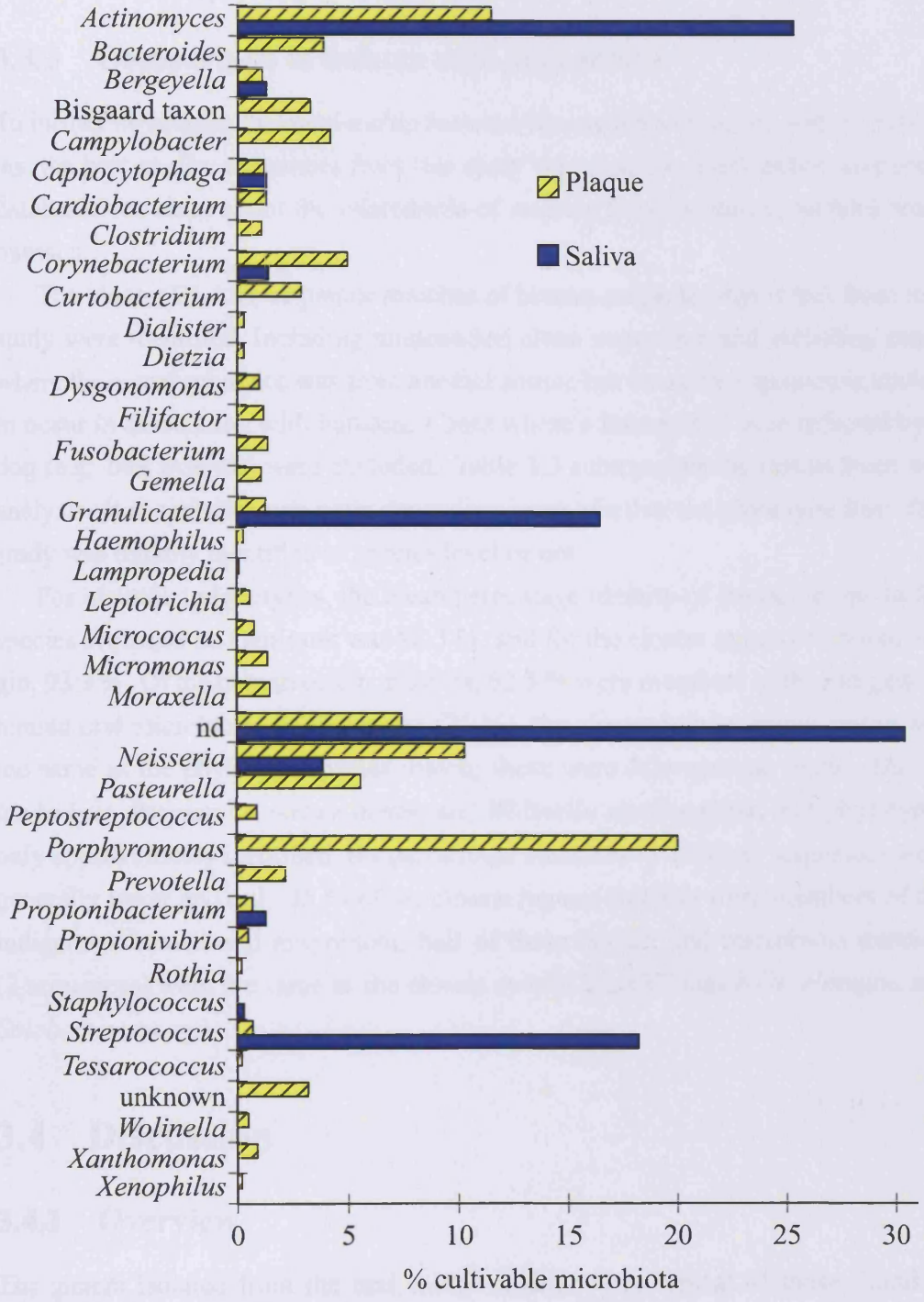


Figure 3.4: Summary of genera isolated from plaque and saliva as mean % total cultivable microbiota (percentages were calculated per sample before averaging). Unknown indicates sequences unrepresented on GenBank, nd indicates that sequence data was insufficient for identification or was not obtained.

3.3.3 Comparison to human oral microbiota

To further investigate the relationship between the human and canine oral microbiotas, the best quality sequences from this study were used to query public sequence databases for clues about the relatedness of isolates from canines to isolates from humans.

The closest BLAST sequence matches of human origin to phylotypes from this study were identified, including unidentified clone sequences and including cases where the actual sequence was from another source but the same organism is known to occur in association with humans. Cases where a human had been infected by a dog (e.g. bite wounds) were excluded. Table 3.3 summarises the results from this analysis. It is split into two parts depending upon whether the phylotype from this study was reliably identified to species level or not.

For identified phylotypes, the mean percentage identity of the phylotype to the species sequence on GenBank was 98.3 %, and for the closest match of human origin, 93.3 %. Of the human origin matches, 62.5 % were members of the indigenous human oral microbiota. In four cases (25 %), the closest human origin match was the same as the phylotype species match; these were *Micrococcus luteus*, *Dialister invisus*, *Propionibacterium acnes*, and *Wolinella succinogenes*. For phylotypes only approximately identified, the percentage identities to database sequences were generally lower and only 25 % of the closest human matches were members of the indigenous human oral microbiota; half of these human oral microbiota matches (2 sequences) were the same as the closest overall BLAST match (*N. elongata* and *Solobacterium* sp.).

3.4 Discussion

3.4.1 Overview

The genera isolated from the oral cavity of dogs were typical of those found in human dental plaque (Paster *et al.*, 2001), including *Actinomyces*, *Porphyromonas*, *Fusobacterium*, *Neisseria*, and *Streptococcus*. Identification of the bacteria to species level, however, often proved difficult. Initial bacterial identifications by DNA se-

	ptype	Species	% ID	Closest sp. from human	% ID
Species level identifications	actino1	*A. canis*	96.7	*Actinomyces* sp.•	91.7
	actino2	*A. canis*	99.4	*Actinomyces* sp.•	92.9
	actino3	*A. bowdenii*	99.7	*A. naeslundii*•	96.4
	actino8	*A. hordeovulneris*	96.1	*A. nasicola*	93.3
	actino9	*A. coleocanis*	96.6	*A. europaeus*	90.4
	arth3	*M. luteus*	98.7	*M. luteus*•	98.7
	berg2	*B. zoohelcum*	95.5	*Flavobacteriaceae* sp.	90.2
	bpp8	*P. cangingivalis*	99.1	*P. levii*	88.5
	bpp9	*P. cansulci*	98.5	*Tannerella forsythensis*•	85.5
	capno1	*C. cynodegmi*	97.4	*Capnocytophaga* sp.	94.3
	dia1	*D. invisus*	99.8	*D. invisus*•	99.8
	nei3	*N. canis*	99.5	*Neisseria* sp.•	93.1
	past1	*P. dagmatis*	98.5	*Haemophilus* sp.	89.9
	prop3	*P. acnes*	99.8	*P. acnes*•	99.8
	strep2	*S. minor*	98.3	*Streptococcus* sp.•	89.5
	wol1	*W. succinogenes*	98.5	*W. succinogenes*•	98.5
	Mean		**98.3**		**93.3**
	ptype	Approx. species	% ID	Closest sp. from human	% ID
Species level identification not possible	abio1	*G. balaenopterae*	91.2	*G. elegans*	81.9
	actino6	*A. hordeovulneris*	94.9	*A. nasicola*	94.2
	bpp4	*B. uniformis*	91.5	*Bacteroides* sp.	98.2
	bpp5	*Bacteroides* sp.	92.4	*Dysgonomonas mossii*	89.9
	bul1	*Solobacterium* sp.	84.6	*Solobacterium* sp.•	84.6
	card1	*C.valvarum*	93.0	*C.valvarum*	93.0
	cory1	*C. bovis*	79.6	*C. accolens*	79.9
	cory4	*C. macginleyi*	94.1	*C. macginleyi*	94.1
	morax1	*M. osloensis*	89.4	*M. osloensis*	89.4
	morax2	*M. cuniculi*	95.1	*M.cuniculi*	95.1
	nei1	*N. dentiae*	96.0	*N. meningitidis*	93.1
	nei7	*N. elongata*	94.4	*N. elongata*•	94.4
	pep3	*Helcococcus* sp.	89.5	*H. sueciensis*	89.5
	prop2	*F.* spumicola*	91.7	*Luteococcus sanguinis*	91.8
	strep4	*S. bovis*	91.8	*S. mitis*•	90.8
	xen1	*X. azovorans*	94.8	*Lautropia* sp.•	92.2
	Mean		**91.5**		**90.8**

Table 3.3: Comparison of sequences found in the canine oral microbiota to sequences on public databases of human origin. The top section lists phylotypes identified to species level and the lower section shows phylotypes less reliably identified. Percentage similarity to isolates from this study are shown in the % ID columns. • indicates bacteria of oral origin. **Friedmanniella*.

quencing revealed a large proportion of bacteria which appeared not to be represented on public databases, so further sequencing was carried out. This eliminated the possibility of poor sequence data spoiling database matches, and showed that many phylotypes were not represented on the databases.

The total bacterial load in the pooled saliva sample was approximately 1.0×10^6 cfu ml^{-1}, yet counts as high a 1.0×10^9 cfu ml^{-1} have been reported for human saliva (Darout *et al.*, 2002). This discrepancy may be explained by loss of viability due to freezing and thawing of the saliva sample prior to analysis, and perhaps also by differences in salivation rate, anatomy, and licking behaviour.

3.4.2 Comparison with human and animal oral microbiota

Marked differences between the plaque bacteria of different animals have been known for over 30 years. Socransky and Manganiello (1971) in their review of the human oral microbiota, for example, note that rodents lack *Peptostreptococcus*, *Bacteroides* (mostly now *Porphyromonas* and *Prevotella*), *Treponema*, *Vibrio*, and *Leptotrichia* species, and beagle dogs seem to have a higher proportion of *Bacteroides melaninogenicus* (now reclassified as several species). Harvey *et al.* (1995) also noted that some bacteria isolated from dogs and cats differ slightly from those found in humans, and that this has led to the proposal for the re-naming of such organisms. For example, *P. gingivalis* of canine and feline origin is catalase-positive, but isolates of human origin are catalase-negative. It has since been realised that the human and animal origin *P. gingivalis* 'biotypes' actually represent distinct species, and the name *P. gulae* has been given to the catalase-positive *P. gingivalis* - like organisms usually isolated from animals (Fournier *et al.*, 2001).

There has certainly been a boom in naming new species in recent years, as evidenced by the first publication dates of named species identified in this study (n=39). Over half of the identified species were first described in the last 24 years[2], and 28 % were first described in the last five years[3].

[2]since 1981
[3]since 1999

General observations

Sequence analyses and literature searches showed that most isolates obtained from dogs in this study were not normally found in the human oral cavity. In addition, the proportions of certain genera in the canine plaque did not match those typically found in human plaque; for example *Streptococcus* species, which are common in human plaque (e.g. *S. sanguis*), comprised less than 1 % of the total cultivable microbiota. Human dental plaque typically contains streptococci at approximately 28 % of the cultivable microbiota (Socransky and Manganiello, 1971). The results from the present study confirm previous reports of low levels of *Streptococcus* species in dogs (Wunder *et al.*, 1976, reported less than 4 % in supragingival plaque), so it seems that this is a fundamental difference between human and canine dental plaque.

Poor representation of streptococci has also been observed in the dental plaque of certain marsupials (Beighton and Miller, 1977), so perhaps humans are unusual in the animal kingdom in this respect. Since streptococci are considered so important in human dental plaque, particularly as primary colonisers, this difference raises the question of what organism may fill an equivalent niche in dogs and other animals. *Granulicatella* species may be able to play such a role as they are closely related to streptococci and have been isolated from several plaque samples in this study, and at a high frequency from the pooled saliva sample (16.5 %).

Although it is not clear whether *Granulicatella* species are able to act as primary colonisers, an *in vitro* study by Pratten *et al.* (2003) detected *G. adjaciens* in a 24 hour old biofilm grown on human dental enamel from a pooled saliva inoculum, but the organism was below the detection limit in the inoculum itself. It is also possible that streptococci in the present study were present in plaque samples at a proportion below the detection limit determined by dilutions producing confluent growth on agar (i.e. approximately 0.5 % of the total viable count). This could be checked by using selective media or by molecular methods such as fluorescent *in situ* hybridisation, however if streptococci are present in very low numbers they are unlikely to be fulfilling the same function as they do in plaque of human origin.

Another important genus in the human oral cavity is *Fusobacterium*, whose species are pleomorphic but often form long filaments able to adhere to most other

oral genera (Kolenbrander *et al.*, 2002). No *Fusobacterium* species were unequivocally identified from the samples used in this study, however a *Fusobacterium nucleatum* - like species (fuso3) was detected, and two species of the closely related genus *Filifactor* (fuso1 and fuso4) were detected. These bacteria were detected at low levels in plaque (2.6 % combined), which is in agreement with previous reports of a low prevalence of *Fusobacterium* species in the healthy gingiva of dogs (Kornman *et al.*, 1981). The phylotype fuso3 is a strong candidate for being a previously undescribed taxon as its closest match, *F. nucleatum*, differs by 3.9 % over 1101 nucleotides, and all valid *Fusobacterium* species are represented on GenBank.

Clinical relevance

Classic human periodontal pathogens including *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans* were not detected from any of the samples used in this study. Similarly, most other species recognised as being important players in human dental plaque were also not detected. It seems unlikely that the difference can be wholly attributed to isolation failure in the present study because many closely affiliated organisms were detected.

A large proportion of the bacteria isolated from plaque and saliva belong to the *Actinomycetaceae* family, and specifically include a range of *Actinomyces* and *Corynebacterium* species, together accounting for almost 40 % of the bacteria isolated (Figure 3.4). These are clearly important and dominant organisms of the canine oral microbiota, and have recently been implicated in canine periodontitis by Takada and Hirasawa (2000), who suggest that these bacteria may play the same role in canine periodontitis as *P. gingivalis* plays in human periodontitis. This suggestion is based upon their findings that the proportion of these genera possessing a trypsin-like activity (TLA) is increased in canine periodontitis sites compared with healthy sites, and this may explain, in part, the absence of *P. gingivalis* from plaque collected in the present study.

Cardiobacterium species were isolated from three different dogs; a cocker spaniel, and two miniature schnauzers which are both known to have a heart murmur. The highest frequency of isolation was from the cocker spaniel at 14 % of the iso-

lated bacteria from that dog, with only about 1 % isolation rate from the miniature schnauzers. This finding is potentially important because *Cardiobacterium* species can cause endocarditis in humans by colonising the heart valves, and it is well known that oral bacteria can routinely gain access to the bloodstream and reach the heart via cuts in the mouth during chewing etc. (for a review of systemic diseases caused by oral microorganisms see Debelian *et al.*, 1994). There are many reports in the literature of this genus (usually the type species, *C. hominis*), which is also found in the human oral cavity, causing infective endocarditis in humans (for a review see Kiwan *et al.*, 2004). There are no previous reports, to the author's knowledge, of this genus being isolated from dogs or implicated in canine endocarditis.

Basic plaque microbiota

Dent and Marsh (1981) conducted a study of the dental plaque of 9 animal species, including several types of monkey, lemurs, a tiger, a genet, a giraffe, and four American cocker spaniels to test the hypothesis that there may be a basic plaque microbial community common to all animals. Results from the dogs agree approximately with those from the present study, in summary as follows (results from this study in brackets): *Streptococcus* spp. 6.1 % (0.7 %, *Granulicatella* spp. in saliva 16.5 %); *Veillonella* spp. 21.6 % (not detected); *Neisseria* spp. 7.2 % (10.3 %); *Actinomyces* spp. 8.8 % (11.6 %); *Fusobacterium nucleatum* 5.8 % (*Fusobacterium* spp. 1.4 %). They proposed that representatives of the genera *Actinomyces*, *Bacteroides*, *Fusobacteria*, *Neisseria*, *Streptococcus*, and *Veillonella* may constitute the basic components of human and animal gingival margin plaque. The data from the present study supports this hypothesis, except that *Veillonella* species were not detected, and *Streptococcus* and *Fusobacterium* species were rare.

In light of this, it may be appropriate to broaden the criteria for describing the basic plaque microbiota beyond the genus level to accommodate the data from this study, and in recognition of the vast array of niches and organisms available as shown in Table 3.4.

Although it has been known for a long time that human and canine oral communities differed, a lack of detailed information relating to the dog has prevented

Criteria	Canine examples	Human examples
Phylum Fusobacteria	*Leptotrichia* sp.	*Fusobacterium nucleatum*
Class Bacilli (Firmicutes)	*Granulicatella* sp.	*Streptococcus sanguinis*
Genus *Neisseria*	*Neisseria canis*	*Neisseria mucosa*
Genus *Actinomyces*	*Actinomyces canis*	*Actinomyces naeslundii*
Phylum Bacteroidetes	*Por. gulae*	*Por. gingivalis*

Table 3.4: Basic components of canine and human dental plaque, modification of the criteria suggested by Dent and Marsh (1981).

any previous detailed comparison. Superficially the habitats afforded by either host seem quite similar, but on the microbial level they are clearly substantially different. If this were not the case, repeated exposure over the long history of companionship would have surely resulted in a larger set of shared oral bacteria between humans and dogs.

3.4.3 Evolution, taxonomy, and ecology

Species boundaries and novel taxa

The recent explosion of named bacterial species is surely due in large part to the discriminating power of comparative 16S rRNA gene sequencing. This enables the modern researcher to determine a reliable indicator of relatedness quickly and cheaply before committing to the difficult process of characterising and naming a new species. Indeed, sequencing technology has advanced so rapidly that it is possible for a modest laboratory to completely sequence 100 16S rRNA genes in a week. Less than 20 years ago, Woese (1987) was looking forward to the time when it might be possible for a well equipped laboratory to achieve this in a year.

The rapidly growing sequence databases are a valuable resource which has simplified the identification of bacteria in the present study greatly, however 44 phylotypes were not identified to species level because good matches could not be found on public databases. Excluding six phylotypes of below average sequence quality, 38 phylotypes are considered likely new taxa (Table 3.2). The GenBank record for the genus of 12 of these candidate new species includes a representative for every validly named species, so these are particularly likely to represent new species. In

cases where the GenBank record is incomplete, no conclusion can be drawn because a named but unsequenced species may turn out to be a good match.

Considering only phylotypes with high fidelity sequence data available (n=32), four phylotypes may represent previously undescribed genera assuming a sequence divergence of 10 % to indicate genus level differences. This level seemed conservative based on the data presented in this chapter, however there is no generally accepted genus level cut-off, and a divergence of just 3 % has previously been suggested (Drancourt *et al.*, 2000). Probable new genera determined in this way are summarised in Table 3.5.

Phylotype	bp	Closest BLASTs	%ID
bul1	655	*Solobacterium* sp. oral clone	84.6
		Bulleidia moorei	85.0
cory1	683	*Corynebacterium bovis*	79.6
morax1	728	Uncultured gamma proteobacterium	90.1
		Moraxella osloensis	89.4
pep3	696	*Helcococcus sueciensis*	89.5

Table 3.5: Summary of phylotypes which may represent new genera based upon an assumed genus level sequence divergence of 10 % in the 16S rRNA gene. For each phylotype the closest BLAST match is shown on the first line, and the closest BLAST match with a reliable identification is shown on the second line (if different).

When comparing bacterial 16S rRNA gene sequences for the purposes of identification, a percentage similarity of less than 97 % is often used to imply that the originating organisms belong to different species, based upon the work of Goebel and Stackebrandt (1994). Whilst the applicability of this cutoff has been over-interpreted in many studies, as noted by Forney *et al.* (2004), it is still a useful indication for inferring probable species boundaries in the rapidly changing field of bacterial taxonomy. In this study, the 97 % rule was applied loosely by taking into account the level of 16S rRNA gene heterogeneity found in particular groups. Species were more often grouped rather than split in order to prevent overestimation of novel species and to simplify the analysis. The results showed clearly that the canine oral microbiota is composed mainly of species distinct from those found in the human, with species from the two hosts differing by almost 7 % in the

16 S rRNA gene on average (Table 3.3, top section).

In this study, many streptococcal isolates could not be differentiated to species level on the basis of 16 S rRNA gene sequences, probably due to the highly conserved 16S rRNA gene sequence in this genus (Kawamura *et al.*, 1995a). Differentiation of these species may be achieved by sequencing a faster evolving gene such as *sodA* (Kawamura *et al.*, 1999).

In addition to methods presently employed and the methods used here, oral biology could benefit greatly from sequencing on a much larger scale, either the organism or the community scale. Whole genome sequencing is no longer a novelty but a powerful tool in biology, and there are at present 206 complete microbial genomes available on GenBank (Benson *et al.*, 2004). In addition, the recent metagenomic analysis of the Sargasso Sea (Venter *et al.*, 2004) has revealed surprising microbial diversity in this supposedly simple environment, and demonstrated the power of shotgun sequencing for metagenome analysis.

Evolution of oral microbial communities

The marked difference between the human and canine oral microbiotas indicated by comparative 16 S rRNA gene sequencing, has implications for the probable pattern of evolution in oral bacteria. The possibility that bacterial 16S rRNA genes acquire mutations at a universal rate with respect to time is attractive because it allows these sequences to be used as molecular clocks.

A universal substitution rate of approximately 1 % per 50 million years (MY) (i.e. approximately 2 % divergence per 50 MY; Woese, 1987) is often quoted in the literature (Clark *et al.*, 2001), and is used as a basis for the following calculations.

Assuming that the oral microbiota of dogs and humans originated from their common ancestor and formed isolated populations at the speciation event, it can be calculated that, based on the present data, the primate carnivore split occurred approximately 175 million years ago (MYA) (Equation 3.1). The fossil record and molecular analyses, however, place the split at only 80 MYA (Kimura, 1987; Li *et al.*, 1990), though the confidence we can place in such datings is unclear (Wayne *et al.*, 1991).

The most likely explanation for this discrepancy is probably that the molecular

evolution rate of bacteria in the mouth is higher than in the bacteria used to calculate the generally accepted rate; these are mostly environmental isolates or insect endosymbionts so it is possible that their comparatively lower growth rates may reduce their rate of molecular evolution. A substitution rate of approximately 2.2 % per 50 MY is required to fit a divergence time of 50 MY and a sequence divergence of 7 % (Equation 3.2).

$$\frac{7\%\ divergence}{2\%\ divergence/50MY} = 3.5 \cdot 50MY = 175MY \tag{3.1}$$

$$\frac{7\%\ divergence}{80MY} \equiv \frac{4.4\%\ divergence}{50MY} \equiv \frac{2.2\%\ substitution}{50MY} \tag{3.2}$$

Note that correcting for multiple substitutions (e.g. by the method of Jukes and Cantor, 1969), will increase the sequence divergence and therefore enlarge the discrepancy slightly. The sequences generated in this study and used for these analyses are of variable length but all include the region downstream of position 357 on the gene, which is a particularly variable region and may account for the large observed sequence divergence. To test this, longer gene sequences were obtained from GenBank for the same bacteria where possible, and used to repeat the comparison. In most cases, the analyses using the present data and using the GenBank data both generated the same closest human origin matches but, where appropriate, a closer match was used for the repeat analysis. The repeated comparison using only GenBank sequences produced a similarity within 0.1 % of the original analysis over an average of 1430 bases per sequence, so it seems that the sequence divergence data are not biased by use of partial sequences in this case.

An alternative explanation is that niche divergence was the driving force for selection of already diverged species, not the driving force of speciation itself. This raises the problem of where the newly selected species came from. Although it seems reasonable to assume that many of them were present in the common ancestor, this appears unlikely when one considers that similar selection events probably occurred for many different animal species, so the ancestral species would need to have had a much more diverse oral microbiota.

Considerations of species boundaries and 16S rRNA gene sequence comparisons inevitably lead to the question of what defines a species, and ultimately this

must come down to an arbitrary and artificial choice because there is a continuum of diversity to categorise. Although 16S rRNA gene comparisons have revolutionised bacterial taxonomy due to their universal critical function, they appear somewhat inadequate for species demarcation, not least because their critical role dictates that only neutral changes are allowed. This means that 16S rRNA gene heterogeneity does not necessarily indicate functional genotypic differences, the very differences that natural selection requires to generate new species, and surely therefore the ultimate taxonomic measure.

The ideal taxonomic measure should consider the maximum amount of information in the easiest way, so a compromise must be found somewhere between whole genome sequencing and Gram-staining, depending upon the project goal. In this case, comparative 16S rRNA gene sequencing was found to be an ideal tool for a preliminary investigation of the canine oral microbiota. It has facilitated simple and reliable identification of known bacteria for which sequences were publicly available, and highlighted others which may warrant further study by a more thorough approach.

3.5 Conclusions

By isolating bacteria from the oral cavity of dogs, and identifying them using comparative 16S rRNA gene sequencing, it has been shown that the oral microbiota of dogs differs significantly from that of humans. In particular, the species found in either host are not likely to be found in the other, but it is thought that other members of the genus in question are likely to fill a similar niche in many cases.

Superficial similarities and practical considerations may have encouraged the use of dogs for models of oral disease in humans, but the results of this study suggest that such experiments are unlikely to yield useful information regarding specific bacterial involvement in such processes.

The surprisingly large sequence divergence between bacterial 16S rRNA gene sequences from humans and dogs seems best explained by a higher than expected rate of molecular evolution in the bacteria of the oral cavity. If this can be confirmed and measured accurately, then oral community comparisons between animals could be used as a method for estimating their evolutionary divergence times in the ab-

sence of more reliable measures.

It would be interesting to complement this work with similar studies from other animals to help establish an understanding of the 'basic oral microbiota', if such a thing really exists. Identification of pathogens in other animals would also be of great value if detailed comparisons could be made to identify the common features of oral pathogens.

Large scale sequencing is surely now the method of choice for comparative biology, and offers a fascinating new perspective for understanding microbial communities in the future. By removing the focus from the individual cell and considering instead the community as a whole functional unit, communities can be defined by their genes rather than their species. When applied to oral communities, this approach may show for example that there is not so much a 'basic oral microbiota', but a 'basic oral gene pool'.

Chapter 4

Culture-independent analyses

4.1 Introduction

The proportion of bacterial species in the human oral cavity which have been cultured is estimated to be approximately 50 % (Paster *et al.*, 2001). Any complete microbial community analysis must therefore include culture independent techniques for the detection of culture resistant bacteria in order to avoid a culture biased result. Popular methods for whole microbial community analysis are frequently based upon detection of bacterial 16S rRNA gene sequences, for example by PCR-cloning or DGGE. In addition, specific bacteria of interest can be searched for by using selective PCR or DNA hybridisation methods (e.g. microarray). In this study, a whole community analysis of canine dental plaque was undertaken by DGGE, and selective PCR reactions were used to detect certain culture-resistant taxa.

DGGE can be used for separating community 16S rRNA gene sequences into a banding pattern on a gel (Muyzer *et al.*, 1993) which can be further analysed by band excision and sequencing.

Culture-resistant bacteria which have been detected previously in the human oral cavity include novel genera related to *Eubacterium* and *Prevotella*, spirochaetes, TM7 bacteria, and OP11 bacteria (Harper-Owen *et al.*, 1999; Dewhirst *et al.*, 2000; Kumar *et al.*, 2003).

Harper-Owen *et al.* (1999) demonstrated the use of PCR to detect uncultured bacteria from periodontal sites by using selective PCR primers to amplify the 16S

rRNA gene of three target phylotypes, followed with validation by sequencing. They detected *Prevotella*-like and *Eubacterium*-like phylotypes representing novel genera, and a novel phylotype belonging to the genus *Prevotella*. The *Eubacterium*-like phylotype was significantly associated with periodontal disease.

Spirochaetes are generally highly resistant to culture, though a few species have been grown in the laboratory under carefully controlled conditions. They are well known members of the dental plaque of humans and animals, and are frequently implicated in periodontal diseases. The diversity of periodontal spirochaetes in humans was recently examined by Dewhirst *et al.* (2000), who selectively amplified spirochaetal 16S rRNA gene sequences from plaque DNA using a universal forward primer with a selective reverse primer. PCR amplicons were then cloned and 500 clones sequenced; phylogenetic analysis clustered the clones into 10 known cultured species and 47 uncultivated species.

The TM7 bacteria are a recently recognized group of clones representing a major lineage of the domain bacteria with no cultivated representatives (Hugenholtz *et al.*, 2001). Members of this candidate division include clones from diverse sources, including a peat bog, activated sludge, and deep sea hydrothermal sediments (López-García *et al.*, 2003). TM7 bacteria have been shown to be widespread in the human oral microbiota (Brinig *et al.*, 2003) by the use of real time PCR and FISH. They have also been detected on the tongue and possibly implicated in halitosis by cloning and sequencing of 16S rRNA gene sequences (Kazor *et al.*, 2003).

This chapter details culture-independent analyses of the canine oral microbiota, in particular, DGGE was used to generate community profiles, and selective PCR was used to detect culture-resistant species.

4.2 Materials and Methods

4.2.1 Template DNA

DNA extractions from plaque samples (Section 2.3) were used as a template for PCR reactions. For DGGE controls, DNA was extracted from the colonies of pure isolates arising from canine oral samples (Section 3.3.1), and from *Tanerella forsythensis* and *Prevotella intermedia* as described by Gafan *et al.* (2005).

4.2.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE was used as a community fingerprinting tool, by amplifying community 16S rRNA gene fragments and separating them on the basis of melting temperature (Muyzer *et al.*, 1993).

PCR primers

DGGE relies on the presence of a GC clamp in the PCR amplicon to prevent the strands from completely melting during electrophoresis. This is achieved by adding a GC rich region to one of the primers, indicated by primer names ending '-gc'. The primers used for DGGE were 357f-gc and 518R (Table 4.1), as described by Ogino *et al.* (2001).

Name	Sequence (5'-3')
357f-gc	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG
	GCACGGGGGGCTCCTACGGGAGGCAGCAG
518R	GTATTACCGCGGCTGCTGG

Table 4.1: PCR primers used for amplification of bacterial 16S rRNA genes for separation by DGGE. F=forward, R=reverse, gc=GC-clamp (GC-clamp sequence underlined), numbers indicate gene position using *E. coli* numbering.

PCR

Extracted DNA was eluted to 30 μl, and 5 μl was used as a template for a touchdown PCR program (Ogino *et al.*, 2001) as shown in Table 4.2. The standard PCR master-mix described in Section 2.10.2 was used with a total reaction volume of 50 μl. The touchdown cycle reduces the melting temperature (Tm) used in successive cycles, effectively reducing the stringency for primer binding. This is a measure intended to maximise the amount of DNA species amplified without causing problems due to random priming, since the composition of the sample is unknown and therefore it can not be guaranteed that the selected primers will be a good match for all of the taxa present.

Repeat	Condition (function)
	95°C 5 min
× 2	94°C 1 min (denaturation) 65°C 1 min (annealing; Tm) 72°C 1 min (elongation)
until Tm=56°C	as above block reduce Tm 1°C each cycle
× 10	94°C 1 min 55°C 1 min 72°C 1 min
	72°C 5 min

Table 4.2: PCR thermocycling parameters used for preparation of community DNA for DGGE fingerprinting.

Gel system and running conditions

DGGE was carried out using a DCODE™ universal mutation detection system (Biorad, Hemel Hempstead, UK) according to the manufacturers instructions. Using pooled canine dental plaque and saliva as a template for a perpendicular gel showed that a denaturing gradient of 40 % to 80 % would be suitable for separating the DNA fragments in samples of canine origin. Wells on each gel were loaded with 30 μl PCR product and 20 μl sucrose loading buffer. Marker lanes containing PCR products from *Tannerella forsythensis* and *Prevotella intermedia* were added at both ends of each gel as described by Gafan *et al.* (2005) to assist alignment and comparison of gels. All gels were run at 60°C for 21 h at 35 v (735 vh).

Gel imaging

Gels were stained for 1 h with Sybr Green (Molecular Probes) in the dark before being photographed under UV light using a gel imaging system with digital camera (Alphaimager, Alpha Immotech, San Leonardo, CA, USA).

4.2.3 PCR detection of culture-resistant bacteria

DNA extractions from plaque samples (Section 2.3) were used as a template for selective PCR reactions designed to selectively amplify the 16S rRNA gene of spirochaetes, TM7, and OP11 group bacteria.

PCR primers

PCR primers previously shown to selectively amplify 16S rRNA gene fragments of the target bacterial groups were used to prime the PCR reactions. Table 4.3 shows primer sequences along with the original publication from which they were taken.

Name	Description	Sequence (5'-3')	ref
C90	*Spirochaetes* reverse	GTTACGACTTCACCCTCCT	1
TM-1177R	TM7 reverse	GACCTGACATCATCCCCTCCTTCC	2
C75	universal forward	GAGAGTTTGATYCTGGCTCAG	1
Bac-8F	universal forward	AGAGTTTGATCCTGGCTCAG	3

Table 4.3: Primers for selective amplification of spirochaete and TM7 16S rRNA genes. 1=Dewhirst *et al.* (2000), 2=Brinig *et al.* (2003), 3=DeLong (1992).

PCR

PCR was performed using the standard mixture described in Section 2.10.2, the thermal cycling parameters were adjusted according to previously published parameters as shown in Table 4.4. PCR reactions used 1 μl template and had a total volume of 50 μl.

Cloning and sequencing

The originating sequences producing positive bands for the TM7 PCR were determined by cloning products into TOPO™ plasmids in *E. coli* (Section 2.13), then sequencing the cloned DNA as described in Section 2.11. Successful insertions were checked for size by amplifying plasmid DNA using the M13 primers which flank the insertion site, and running the product on an agarose gel with a size marker (Section 2.10).

Repeat	Condition
Spirochaetes (C75 + C90), ref 1	
× 30	94°C 45 s 60°C 45 s 72°C 90 s (+5s)
	72°C 15 min
TM7 (Bac8F + TM7-1177R), ref 2	
	96°C 3 min
× 35	94°C 1 min 64°C 1 min 72°C 2 min
	72°C 3 min

Table 4.4: Thermocycling paramters for selective amplification of *Spirochaete* and TM7 16S rRNA genes. Primer names are shown in brackets. 1=Dewhirst *et al.* (2000), 2=Brinig *et al.* (2003).

4.3 Results

4.3.1 Community profiling (DGGE)

Pure cultures

DNA amplicons from a selection of pure culture isolates (Section 3.3.1) were run in separate lanes to check that reasonable separation of taxa could be achieved. It can be seen from the first 11 lanes of Figure 4.1 that most of the taxa migrated different distances and would therefore be easily resolved in a defined community. Some taxa appear at first glance to have co-migrated the same distance, but a careful examination aided by pixel intensity measurements showed that these too could be resolved. It can be seen in this way that the pairs of almost co-migrating taxa are each closely phylogenetically related; strep2/lac1 (*Streptococcus minor* / *Streptococcus* sp.), bpp3/bpp6 (*Prevotella heparinolytica* / *Porphyromonas gulae*), and

bpp11/bpp8 (*Porphyromonas macacae* / *Porphyromonas cangingivalis*).

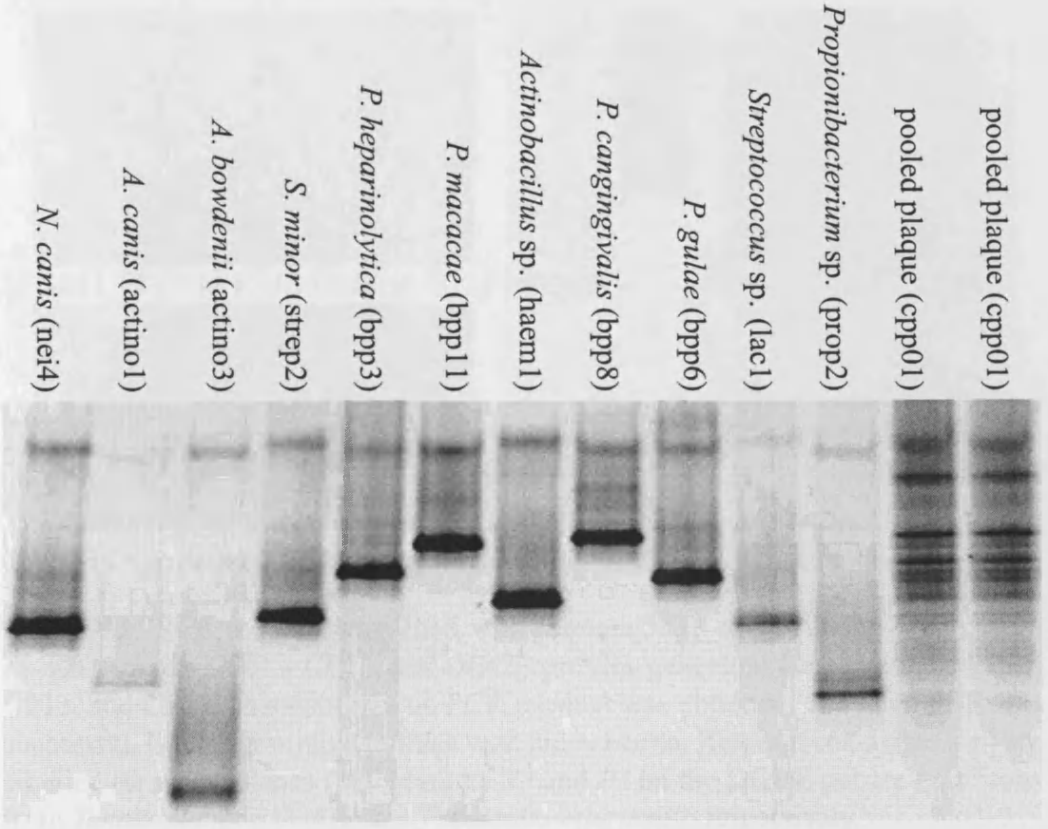


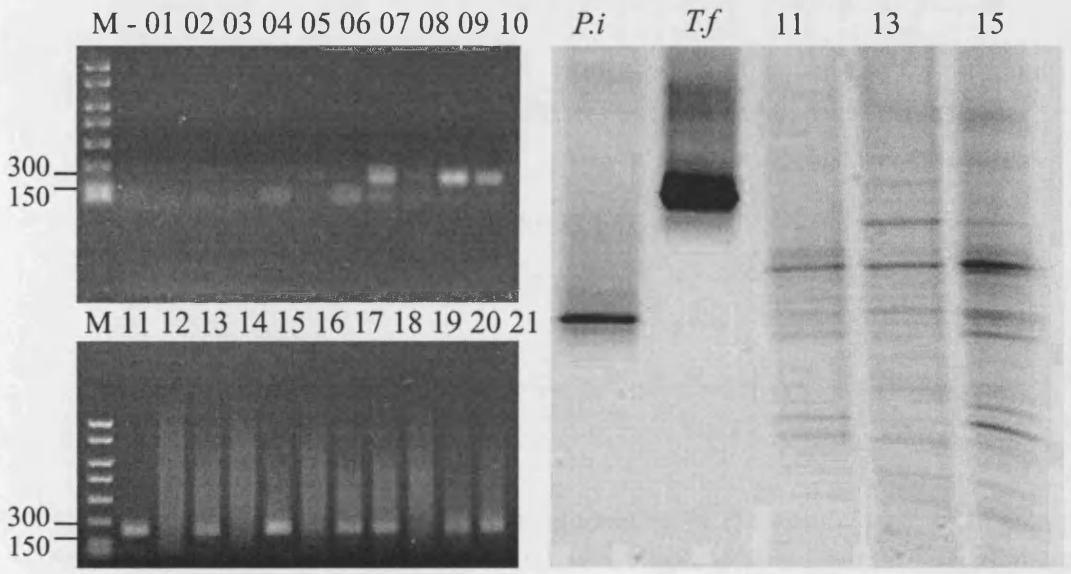
Figure 4.1: DGGE pure isolates and pooled plaque.

Pooled plaque samples

The duplicate pooled plaque samples shown in the last two lanes of Figure 4.1 produced identical profiles, with 13 distinct bands clearly resolved despite loading different amounts of product in each well (5 μl or 10 μl).

Individual plaque samples

In most cases, PCR using plaque DNA extract as template, with the primers 357F and 518R-gc produced little or no product (Figure 4.2 a), however when PCR products were obtained they produced DGGE profiles with up to 14 bands (Figure 4.2b).



(a) PCR products from canine plaque.

(b) DGGE profiles from PCR products.

Figure 4.2: PCR products amplified with primers 357F and 518R-gc from canine plaque samples CP01 - CP21, and DGGE profiles generated from products CP11, CP13, and CP15. In most cases no PCR product was obtained, but when PCR was successful, DGGE generated profiles with many bands. Relevant band sizes (bp) are labelled for marker lanes (M). Markers *T.f* and *P.i* on the DGGE gel are amplicons from *Tannerella forsythensis* and *Prevotella intermedia* respectively.

4.3.2 Culture-resistant bacteria

The presence of *Spirochaetes* and TM7 group bacteria was indicated by products of the predicted size after selective PCR using template DNA from canine plaque samples (Table 4.5). It is possible for a product of the correct size to be produced in the absence of the target organism if other organisms share the primer sequence, or if similar sequences in the mixture bind non-specifically to the primers. A selection of PCR amplicons were therefore checked by cloning and sequencing according to the methods described in Section 2.13.

(a) Susie (samples CP11 - CP33)

										maxilla											
Right	110	109	108	107	106	105	104	103	102	101	201	202	203	204	205	206	207	208	209	210	Left
GI				0							0	0	0	0	0	0	0	0	0	0	
PPD				0							3	3	0	3	5	3	5	5	0	0	
TM7				-							-	+	-	+	-	-	+	+	-	-	
SP				-							-	-	+	+/-	+	+	+	+	-	-	

										mandible												
Right	411	410	409	408	407	406	405	404	403	402	401	301	302	303	304	305	306	307	308	309	310	311	Left
GI						0		3	3	0			0	0	0	0	0	0	0	0	0	0	
PPD						3		0	0	0			0	0	0	0	0	0	0	0	0	0	
TM7										+			-	+	-	+	+	-	+	+	-	+	
SP										+			+	+	+	+	+	+	+	+	-	+	

(b) Dica (samples CP34 - CP57)

										maxilla											
Right	110	109	108	107	106	105	104	103	102	101	201	202	203	204	205	206	207	208	209	210	Left
GI				2			3			0	0	2	3	0							
PPD				4			5			0	3	0	3	4							
TM7				-			-			-	-	-	-	+/-							
SP				-			-			+	-	-	-	-							

										mandible												
Right	411	410	409	408	407	406	405	404	403	402	401	301	302	303	304	305	306	307	308	309	310	311	Left
GI	0	0	3		3	2	0	2	1	0	0	0	0	1	2	2	3	3		3	2	0	
PPD	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	
TM7	-	-	-		-	-	-	-	-	-	-	-	-	-	+	+	-	-		+	-	-	
SP	-	-	-		-	-	-	-	-	-	-	-	-	-	+	+	-	+/-		-	-	-	

Table 4.5: TM7 and *Spirochaetes* (SP) detected by PCR from the teeth of two dogs. Presence or absence of a PCR amplicon indicating detection from the buccal plaque of each tooth is indicated by + or - respectively, blanks indicate that no plaque sample was available for testing. Gingivitis index (GI), and periodontal probing depth (PPD, in mm) clinical parameters are also shown.

Cloning

Cloning of PCR products was attempted for two of the positive TM7 bands, corresponding to plaque samples CP12 (Susie 202) and CP18 (Susie 208). Successful insertion of DNA into the clones was indicated by colony growth on ampicillin- and kanamycin-containing agar, and 20 colonies were randomly picked for each PCR product. Colonies were used as a template for PCR using M13 primers which flank the insert point on the plasmid. The product from this PCR was sized on an agarose gel to check that the insert was the correct size, and then the products were sequenced.

The gel image in Figure 4.3 shows the first five M13 clone PCR products for the two TM7 positive bands selected, and it can be seen that products of the correct size (approximately 1200 bp) were obtained for CP18, but weak products of the wrong size were produced for CP12.

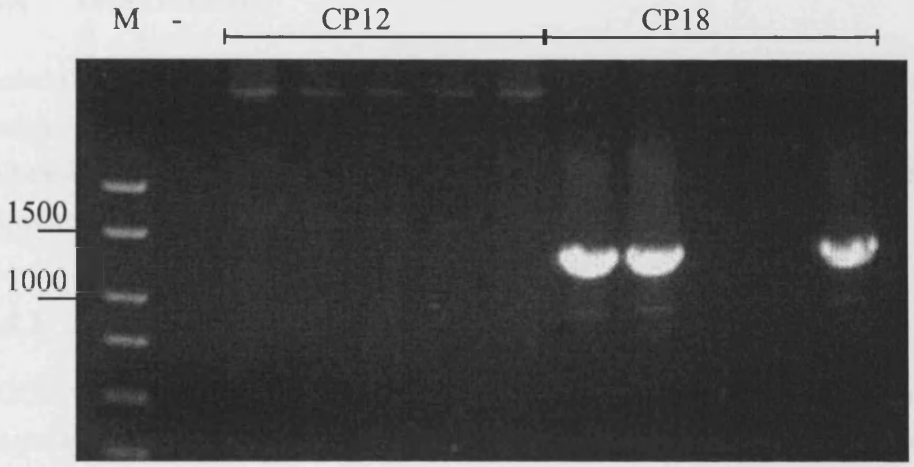


Figure 4.3: M13 insert PCR products from clones of amplified TM7 DNA of two plaque samples. Five clones from each sample are shown, along with a negative control (-). Relevant band sizes (bp) are labelled for the marker lane (M).

Sequencing

The sequences of five different clones from CP18 were submitted to GenBank, with accession numbers DQ156980-DQ156984. BLAST searches on GenBank for all sequences identified the originating organism as *Wernerella denticanis*, not a TM7 group bacterium. A BLAST search using the longest sequence obtained showed that the most closely related sequence on GenBank[1] was *Wernerella denticanis*, however this is not at present a validly published name. Table 4.6 shows the best five matches found by the BLAST search.

Accession	Description	Score	E-value
AY560020	*Wernerella denticanis* strain B106 16S ...	1501	0.0
AY134906	*Bacteroidetes* sp. oral clone FX069 16S ...	1421	0.0
AF530302	Uncultured bacterium clone cadhufec059h7 ...	396	e-107
L16496	*Bacteroides splanchnicus* NCTC 10825 ...	379	e-102
AY916248	Uncultured bacterium clone NN84 16S ...	361	2e-096

Table 4.6: BLAST search results for CP18 clone 11. The score is a raw measure of sequence similarity and the E-value indicates the probability of the match occurring by chance.

[1] 10 March 2005

4.4 Discussion

Molecular analysis techniques allow bacteria present in a sample to be detected and studied without the need for growing them. This can help fill the gap left in the culture-based analysis caused by culture-resistant bacteria in the sample, or by bias introduced by culture-based methods.

4.4.1 Community profiling

DGGE was used to generate bacterial community fingerprints from canine dental plaque to allow comparison of samples from, for example, different sites, different dogs, or *in vitro* models. Unfortunately, problems encountered in the initial amplification step resulted in only a few profiles being obtained; too few to make many meaningful comparisons.

For most plaque samples, amplification for DGGE using primers 357F-gc and 518R failed. The reason for this was suspected to be that there was insufficient template DNA available, and this supposition was supported by success in amplifying the pooled plaque sample which contained a larger amount of plaque than the other samples. Successful amplification using 27F and 1492R primers, however, suggested that there was template DNA available but that the DGGE primers were not as efficient. This could be due to poorer specificity of the DGGE primers used, and the GC clamp may sterically interfere with the PCR.

Community profiles were successfully generated from individual and pooled plaque samples, and separation was demonstrated for pure isolates obtained from canine plaque. These results showed that with sufficient DNA available, DGGE can be successfully used to generate a profile from canine plaque bacteria, and that a selection of individual taxa will produce resolvable single bands.

Up to 14 bands were detected from individual plaque samples, which compares to a maximum of 22 phylotypes detected from a single sample by culture (Section 3.3.1). Given that approximately 50 % of the oral microbiota is expected tot be unculturable by the methods used in this study, one might expect a perfect culture-independent study to have discriminated approximately 50 phylotypes from canine plaque samples based upon the results from Chapter 3. The results from this study

therefore suggest that DGGE performed on canine plaque samples was unable to resolve all of the species (or phylotypes) present. Several factors could explain this result which is similar to that found previously for DGGE profiles of human dental plaque (Gafan *et al.*, 2005). In particular, co-migration of different species may occur, primer bias may prevent amplification of certain species, and some species present in small number may not be detected because they are out-competed during the PCR. Other difficulties with the use of DGGE include the reproducible preparation of denaturing gels, and collection of DNA from bands of interest.

Some of the problems with DGGE could potentially be solved if a capillary gel was used in combination with a temporal temperature gradient; a higher resolution can be achieved on capillary gels such as those used on DNA sequencing machines, and a temperature gradient would probably be easier to control than a chemical denaturant gradient. In addition, it is possible to have capillary systems set up to collect DNA fragments emerging from the capillary, thus allowing for phylogenetic information to be easily obtained from peaks of interest. Another advantage of such a system is that control markers could be placed in the capillary with the sample and the resulting electropherograms could be automatically calibrated for easy comparison, however this technology is not yet commercially available.

4.4.2 Culture-resistant bacteria

Both spirochaetes and TM7 bacteria were detected by PCR from canine plaque samples, but confirmation of amplicon identities was not obtained so no conclusion can be drawn from this result. Some difficulty was experienced whilst trying to clone the PCR products, so it was possible to obtain sequence data for only one TM7 clone, from plaque sample CP18. A BLAST search showed that this was not a bacterium belonging to the TM7 candidate division, but was closely related to two sequences on GenBank belonging to the *Bacteroidetes* phylum.

The closest database match was described as *Wernerella denticanis*, which has also been described as *Porphyromonas denticanis* (Hardham *et al.*, 2005), found to be among the most frequently isolated black-pigmented anaerobic bacteria (BPA) from canine periodontitis, along with *P. gulae* and *P. salivosa*. A valid description of this organism, to be named *Odoribacter denticanis* is presently in press (Hard-

ham *et al.*, In Press). The next closest match to the sequence presented here and to *Odoribacter denticanis* (Hardham *et al.*, 2005) was to an oral *Bacteroidetes* clone (Paster *et al.*, 2002). The closest match of both of these sequences to a speciated bacterium was to *Bacteroides splanchnicus*, and it has been proposed that this organism also should be moved to the new genus *Odoribacter* (Hardham *et al.*, In Press).

No phylotypes similar to *Odoribacter* species were detected in the culture based part of this study (Chapter 3), but no attempt was made to isolate bacteria from the same sample so it can not be determined whether this was due to a failure of the cultural technique or because the organism was rarer or absent in the samples subjected to culture-based analysis.

The importance of carefully validating PCR conditions for detection of specific DNA species in a mixture has been noted previously by Harper-Owen *et al.* (1999), who in a similar study found that their selective PCR for uncultured oral bacteria also amplified unwanted targets.

Since a large portion of most polymicrobial samples, including dental plaque, is unknown and resistant to culture, such validation is difficult. In this study, the chosen approach was to use previously published and validated PCR protocols and then to re-validate by sequencing PCR amplicons rather than testing a selection of positive and negative control templates. Unfortunately, poor cloning efficiency of amplicons severely limited the value of this approach; however the original authors validated their protocols so it is reasonable to expect that some of the TM7 and spirochaete amplicons obtained were indeed generated from DNA of the target organisms.

4.5 Conclusions

Analysis of canine plaque samples by DGGE has shown that this bacterial community is amenable to such analyses in the same way as human dental plaque, however sample volume seems to have been a limiting factor in whether sufficient DNA was obtained from samples for amplification using the primers 357F-gc and 518R. When amplification succeeded, profiles with up to 14 bands were produced.

Selective PCR reactions generated amplicons using primers specifically targeted

for TM7 bacteria and spirochaetes, and it was attempted to confirm the sequence of these amplicons but cloning difficulties meant that sequences were obtained for only one TM7 amplicon. These sequences showed that the PCR reaction had amplified DNA from a species of a recently described new genus, *Odoribacter denticanis*. This result shows that the TM7 selective PCR is not entirely specific for the TM7 candidate division, but it does not exclude the possibility that some of the amplicons were produced from TM7 bacteria.

Chapter 5

Coaggregation

5.1 Introduction

Coaggregation is the term used to describe the adhesion of genetically distinct microbial cells to each other in a suspension (Kolenbrander and London, 1993), and autoaggregation describes the same phenomenon between genetically identical cells. These processes, in conjunction with adhesion to a substratum, are thought to be important factors in the development of bacterial biofilms. Coaggregation may have evolved hand in hand with structural and metabolic co-dependencies which in turn facilitate the development of complex biofilm systems such as dental plaque. This possibility is supported by the work of Bradshaw *et al.* (1998) who showed that certain bacterial aggregates could permit the persistence of obligate anaerobes in aerated cultures.

Gibbons and Nygaard (1970) were the first to realise the potential importance of cell to cell adhesion in the development of dental plaque, and develop a method for measuring interbacterial aggregation, which is now more commonly called coaggregation. They used this method to assess 23 strains of prominent plaque bacteria for coaggregation and found that 18 of them participated in coaggregation reactions; a total of 23 interactions were observed from the 253 pairs tested. The effect of pH (5.0 - 9.0) and growth phase were also examined and found to be unimportant. This initial work has been continued over the following years by many workers, who frequently assess coaggregation by the convenient visual assay described by Cisar

et al. (1979), which uses a scoring system from 0 - 4.

Coaggregation research has been strongly focused on bacteria isolated from human dental plaque, but has recently also been shown to occur in water systems, leading to the suggestion that it may be a universal phenomenon among biofilm-forming bacteria (Rickard *et al.*, 2000). Coaggregation studies using bacteria from the canine oral microbiota will allow comparison with the large amount of data relating to the human oral microbiota, and may help to identify important interactions for the development of canine dental plaque.

For this study, initial coaggregation assays were performed using conditions published for use with bacteria isolated from the human oral microbiota. The conditions were then adjusted to more accurately replicate the canine *in vivo* conditions. Finally the whole cultivable bacterial community from a single plaque sample (CP06) was assayed in duplicate using the canine-adapted assay conditions.

5.2 Materials and Methods

5.2.1 Bacterial isolates

All bacteria used in this study were isolated from the dental plaque of dogs as described in Section 3.2.2. The phylotypes and identities of bacteria used in coaggregation studies are listed in Table 5.1.

5.2.2 Culture preparation

Bacterial cultures were grown in 20 ml static brain heart infusion broths (Oxoid) for 18 hours or 40 hours with supplements supplied as required. The cultures were grown for long enough to produce a dense suspension in order to supply a sufficient concentration of cells. The cells were harvested by centrifugation (4000 RCF for 10 min at 4°C) and resuspended in 5 ml coaggregation buffer (Table 5.2) three times. The cell density of each suspension was then adjusted to 1.0 at 600 nm against sterile buffer.

Phylotype	Identification
abio1	*Granulicatella* sp.
actino1	*Actinomyces canis*
actino2	*Actinomyces canis*
actino3	*Actinomyces bowdenii*
actino4	*Actinomyces* sp.
actino8	*Actinomyces hordeovulneris*
berg1	*Bergeyella zoohelcum*
bpp10	*Porphyromonas* sp.
bpp11	*Porphyromonas macacae*
bpp4	*Bacteroides* sp.
bpp6	*Porphyromonas gulae*
bpp7	*Porphyromonas canoris*
bpp8	*Porphyromonas cangingivalis*
bpp9	*Porphyromonas cansulci*
card1	*Cardiobacterium* sp.
cory2	*Corynebacterium* sp.
cory3	*Corynebacterium* sp.
fuso3	*Fusobacterium* sp.
haem1	*Haemophilus* sp.
lac1	*Streptococcus* sp.
lep1	*Leptotrichia* sp.
fuso4	*Filifactor villosus*
nei1	*Neisseria* sp.
nei3	*Neisseria canis*
nei4	*Neisseria canis*
nei5	*Neisseria weaveri*
nei8	*Xanthomonas* sp.
pep1	*Peptostreptococcus anaerobius*
prop2	*Propionibacterium* sp.
staph1	*Staphylococcus epidermidis*
strep2	*Streptococcus minor*

Table 5.1: Identities of bacterial phylotypes used in coaggregation assays.

Supplements

In cases where bacteria grew poorly in broths, or if a specific nutrient requirement was known, supplements were added as appropriate.

The supplements used (not necessarily all together) were 5 μg ml^{-1} haemin (Sigma) (stock dissolved in 1M NaOH), 0.5 μg ml^{-1} menadione (Sigma) (stock dissolved in 70 % ethanol), 750 ng ml^{-1} cysteine hydrochloride (BDH), and 5 μg ml^{-1} yeast extract (Oxoid). Haemin and menadione were filter-sterilised and added to sterile broths, cysteine hydrochloride and yeast extract were added to broths before autoclaving.

5.2.3 Standard coaggregation assay

Initially, a coaggregation assay was performed on selected bacterial isolates from the cultural study according to methods described by Cisar *et al.* (1979) to identify coaggregating pairs of bacteria from the canine oral microbiota. Coaggregation buffer was made at pH 8.0 buffered with 1 mM TRIS, and its main ingredient was 0.15 M NaCl, see Table 5.2 for detailed composition. The assay was carried out as follows:

1. Mix 0.2 ml of each pair in a Durham tube and vortex mix for 10 s.
2. Stand mixtures at room temp for 1-2 h.
3. Mix again (10 s vortex) and score.
4. Stand overnight (room temperature), mix again and check score.

Scoring criteria:

0. No visible aggregates.
1. Small uniform coaggregates in suspension.
2. Definite coaggregates, easily seen but suspension remained turbid without immediate settling of coaggregates.
3. Large coaggregates which settled rapidly leaving some turbidity in the supernatant fluid.

4. Clear supernatant fluid and large coaggregates which settled immediately.

All coaggregation assays were set up as a grid with identical isolates on each axis so that each possible pair cross was performed twice, and autoaggregation crosses were also duplicated. Assays were performed blind with isolate numbers identifying the crosses, and at the end of each scoring session the results grid was examined for any discrepancies between the duplicate crosses. When a discrepancy was found the pair were re-checked and re-scored; invariably the discrepancy was due to score interpretation difference rather than an actual difference in reaction.

The effect of autoaggregation was corrected for by subtracting the highest autoaggregation score of the two partners from the coaggregation score as described by Rickard *et al.* (2003). Although this method has the undesirable effect of masking some genuine interactions, it avoids the possibility of declaring false positive interactions caused by autoaggregation, which is considered a worse situation. The visual assay was chosen to assess coaggregation in preference to using a spectrophotometer because the visual assay is easier to perform, and Cisar *et al.* (1979) found that comparable results were obtained with both methods.

5.2.4 Buffer time course assessment

A time course experiment was performed to assess the influence of salts composition on coaggregation experiments. Four different salts compositions were compared, all based on 0.01 M HEPES at pH 8.0; these were coaggregation buffer salts, 0.15 M NaCl, canine artificial saliva (CAS) salts, and no salts. Suspensions containing a mixture of four bacterial species were followed over six hours by measuring optical density at 600 nm. The bacteria used were *Corynebacterium felinum*, *Fusobacterium nucleatum* - like, *Leptotrichia* sp., and *Porphyromonas gulae* (phylotypes cory2, fuso3, lep1, bpp6).

5.2.5 Canine-adapted coaggregation assay

To adapt the standard coaggregation buffer for bacteria isolated from dogs, the pH was lowered to 7.5 and the salts composition was adjusted, according to measurements made at the Waltham Centre for Pet Nutrition. In addition, the pH buffer

Ingredients	Standard	Adapted
TRIS	0.001	0.0
HEPES	0.0	0.01
KCl	0.0	0.02
NaCl	0.15	0.04
$CaCl_2$	0.0001	0.001
$MgCl_2$	0.0001	0.0
Total Salts	0.1502	0.061

Table 5.2: Molar quantities of ingredients for standard coaggregation buffer, and coaggregation buffer adapted for use with bacteria isolated from the canine oral cavity. Buffers also contain 0.02 % (w/v) sodium azide.

was changed to HEPES which is more biologically compatible than TRIS (Good *et al.*, 1966), and the buffer strength was increased to 0.01 M because the pH of the standard buffer was observed to be unstable.

5.2.6 Transmission electron microscopy (TEM)

Some of the coaggregating pairs of bacteria were selected for examination by transmission electron microscopy (TEM) using methylamine tungstate as a negative stain to visualise the bacterial aggregates.

Cells were prepared for TEM by removing 1 ml of the coaggregation assay cell suspension and centrifuging at 7200 RCF for two minutes. The supernatant was removed and replaced with sterile distilled water, then the cells were resuspended and pelleted again in the same manner. The supernatant was removed and the cells resuspended in 200 μl sterile distilled water, thereby concentrating the cells five-fold and removing the coaggregation buffer which was found to be incompatible with TEM. Cells prepared in this way were visualised by negative staining as described in Section 2.6.3.

5.3 Results

5.3.1 Standard coaggregation assay

Initial experiments used standard coaggregation conditions as described by Cisar *et al.* (1979) to make 224 unique test crosses with 28 different bacterial phylotypes obtained from the canine oral microbiota. Repeats of 10 % of the crosses were performed, and 95 % yielded identical results after correction for autoaggregation. These experiments revealed a total of 35 different coaggregation interactions between 15 bacterial phylotypes isolated from the canine oral microbiota as summarised in Table 5.3.

5.3.2 Buffer time course assessment

A four way coaggregation reaction was followed by optical density measurements over six hours to determine whether buffer composition was an important factor in coaggregation reactions. The results from this experiment are shown in Figure 5.1, and it can be seen that the optical density change over time had a different profile depending on the composition of the cell suspension buffer. The most rapid reduction in optical density was observed with the cells suspended in distilled water which lost almost 80 % of their absorbance after one hour. By comparison, at the same time point cells in canine-adapted buffer lost approximately 55 % of their absorbance, and cells in standard coaggregation buffer and sodium chloride both lost approximately 40 % of their absorbance. After the first hour, the optical density of the suspensions stabilised and declined very gradually for the remainder of the experiment, however the canine-adapted buffer continued to reduce it's absorbance at a slightly faster rate.

5.3.3 Canine-adapted coaggregation assay

Duplicate coaggregation experiments performed on the cultivable oral microbiota from a single dog, using canine-adapted conditions were in agreement for 74 % of cases. All possible crosses of 15 phylotypes were performed (120 crosses), revealing a total of 8 different coaggregation reactions involving 8 different isolates

	abio1 (*Granulicatella* sp.)	actino1 (*A. canis*)	actino2 (*A. canis*)	actino3 (*A. bowdenii*)	actino4 (*Actinomyces* sp.)	actino8 (*A. hordeovulneris*)	berg1 (*B. zoohelcum*)	bpp10 (*Porphyromonas* sp.)	bpp11 (*P. macacae*)	bpp4 (*Bacteroides* sp.)	bpp6 (*P. gulae*)	bpp7 (*P. canoris*)	bpp8 (*P. cangingivalis*)	bpp9 (*P. cansulci*)	cory2 (*Corynebacterium* sp.)	cory3 (*Corynebacterium* sp.)	fuso3 (*Fusobacterium* sp.)	haem1 (*Haemophilus* sp.)	lac1 (*Streptococcus* sp.)	fuso4 (*F. villosus*)	nei1 (*Neisseria* sp.)	nei3 (*N. canis*)	nei4 (*N. canis*)	nei5 (*N. weaveri*)	pep1 (*P. anaerobius*)	prop2 (*Propionibacterium* sp.)	staph1 (*S. epidermidis*)	strep2 (*S. minor*)
abio1	0	0		1	1	1			0	1	1		0	0		0		0		1		1			0	0	0
actino1		0		0	0	0			0	0	0	0	0	0		0	1		0		0		1			0	1
actino2			0				0				0				0	0	0	0		0	0			0			
actino3				0	0	0			0	0	0		1	0		0		0		0						0	1
actino4					0	0			0	0	0		1	0		0		0		0						0	1
actino8						0			0	0	0		1	0		0		1		1						0	1
berg1							0				0				1	0	0	0		0	0			0			
bpp10								1	0	0			0			1						0		0			
bpp11									0	0	1		0	0		0	0	0		0		0	0	0		0	0	0	0
bpp4										0	0		1	0		0	0	0		0		0	0	0		0	0	0	1
bpp6											0		0	0	0	0	0	0	1	0	1		0	0		0	1	0
bpp7												1				1							0				0
bpp8													0	0		0		0		0		0		0		0	0	0
bpp9														0		0	1		0		0	0	0		0	0	0	0
cory2															0	0	0	0		0	0			0				
cory3																0	0	0	0	0	0		0	0		0	0	1
fuso3																	1	0		0	0	0	1	0	1			1
haem1																		0		0	0			0				
lac1																			1		0		0			0	0	0
fuso4																				1	0			0				
nei1																					0		0	0		1	0	0
nei3																						0		0				
nei4																							0			1	0	1
nei5																								0				
pep1																									0			
prop2																										1	0	0
staph1																											0	0
strep2																												1

Table 5.3: Summary of coaggregation interactions corrected for autoaggregation, detected between bacteria from experiments 1-6 using standard conditions. Blanks indicate crosses which were not tested.

(Figure 5.2).

5.3.4 Transmission electron microscopy of coaggregates

Several isolates from the canine-adapted coaggregation assay were further examined by transmission electron microscopy using a negative stain to highlight cell surface detail. In each case, single species suspensions were examined and cell clumping appeared less frequent than in the mixed populations, but the difference was not quantified.

Haemophilus sp. (haem1)

The strong autoaggregator haem1 (*Haemophilus* sp.) was viewed by itself and was found to be a highly fimbriated bacterium (Figure 5.3). Large aggregates were present but some of the bacteria were in isolation.

Leptotrichia sp. and *Actinomyces bowdenii* like (lep1 and actino3)

Lep1 (*Leptotrichia* sp.) appeared as predominantly isolated slender rods which were poorly stained, whilst actino3 (*Actinomyces bowdenii*) cells were isolated or clumped short thick rods which mostly took up the negative stain. This staining distinction facilitated discrimination of cells in the mixed population by TEM, and coaggregations between the two cell types were clearly observed (Figure 5.4), despite not being detected by the assay. Actino3 cells frequently had irregularities on the cell surface which looked as though debris from the medium may have adhered to the cells.

Fusobacterium nucleatum like sp. and *Corynebacterium felinum* like sp. (fuso3 and cory2)

Fuso3 (*Fusobacterium* sp.) cells were large rods which stained variably, cory2 (*Corynebacterium felinum* like sp.) cells formed shorter rods which stained more darkly and also had a thick coat which stained variably. In mixed populations cory2 could be seen as darker and shorter cells coaggregating with the lighter coloured fuso3 (Figure 5.5).

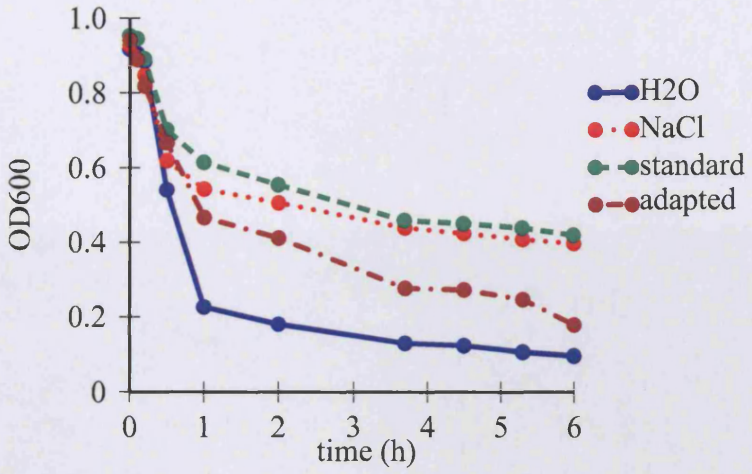


Figure 5.1: Coaggregation time course experiment following the optical density of four way coaggregation mixtures made in different suspension media.

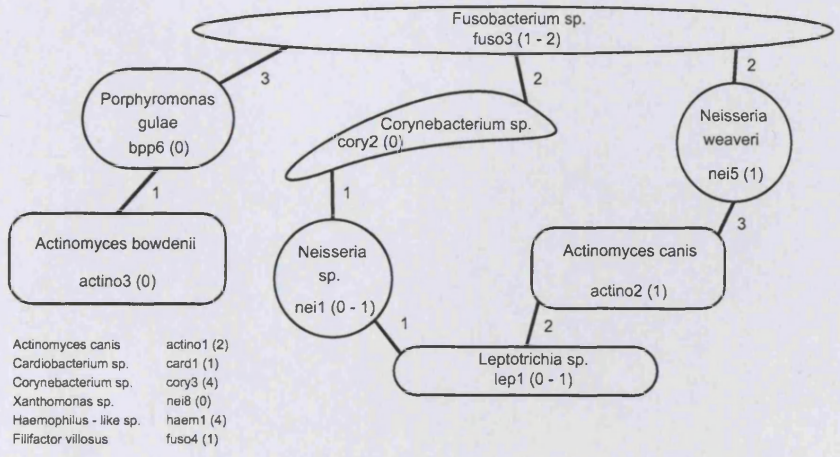


Figure 5.2: Summary of autoaggregation and coaggregation interactions detected between bacteria from a single canine plaque sample using canine-adapted conditions. Numbers within cells represent autoaggregation scores, numbers on lines indicate coaggregation scores. Bacteria for which no coaggregation interactions were detected are listed at the bottom along with their autoaggregation scores.

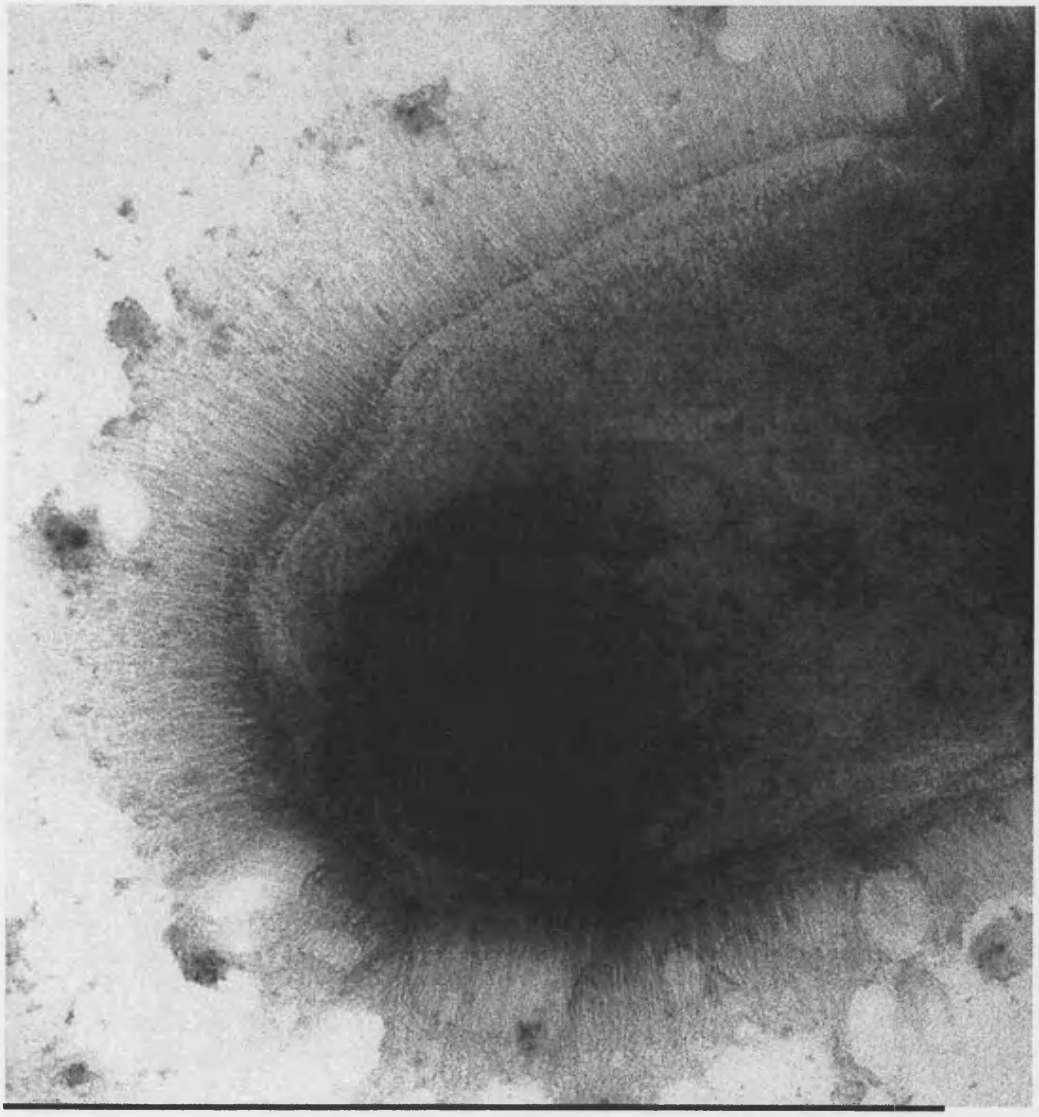
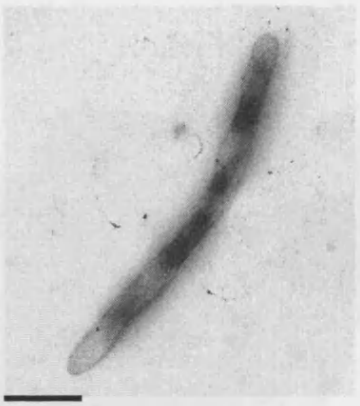
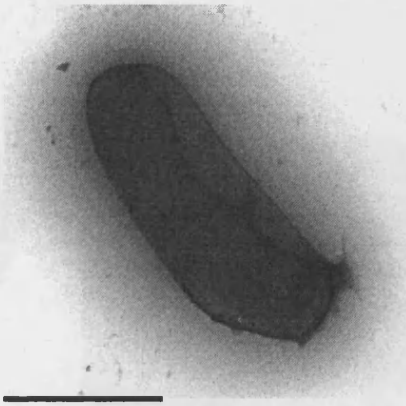


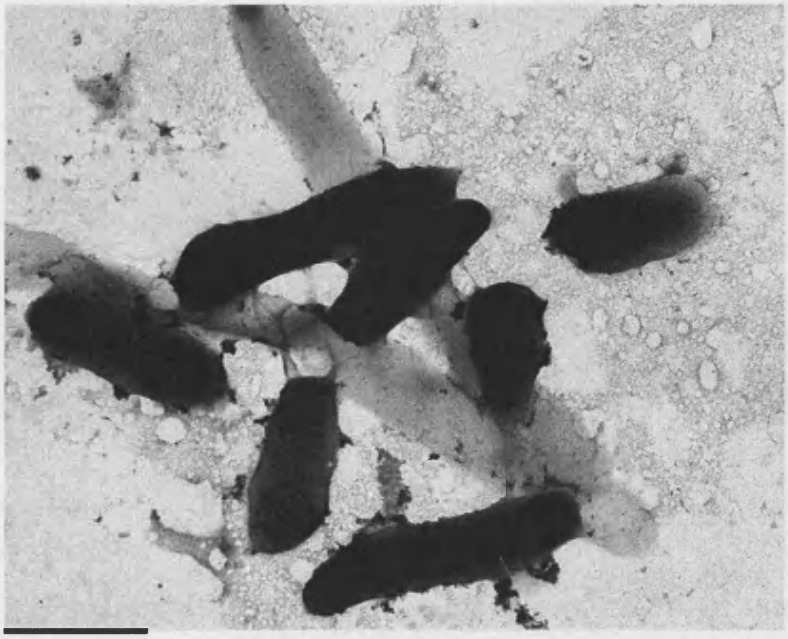
Figure 5.3: Negative-stained TEM of *Actinobacillus* sp. (Haem1). Scale bar indicates 1 μm.



(a) *Leptotrichia* like (lep1).



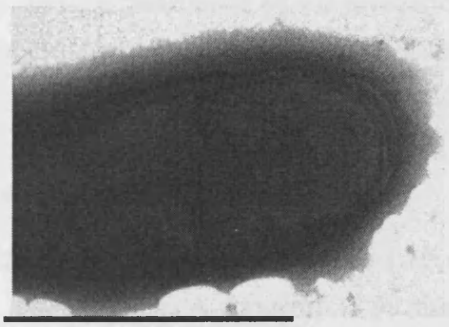
(b) *Actinomyces bowdenii* (actino3).



(c) Coaggregate of lep1 and actino3.

Figure 5.4: Negative-stained TEM of *Leptotrichia* like sp. and *Actinomyces bowdenii*, and a mixed coaggregate. Scale bars indicate 1 μm.

(a) *Fusobacterium* sp. (fuso3)



(b) *Corynebacterium* sp. (cory2)

(c) Coaggregate of fuso3 and cory2.

Figure 5.5: Negative-stained TEM of *Fusobacterium* sp. and *Corynebacterium* sp., and a mixed coaggregate. Scale bars indicate 1 μm.

5.4 Discussion

Using standard coaggregation conditions, 35 unique interactions were detected from 224 crosses (16 %) of bacteria from several dogs. Using canine-adapted conditions, eight unique interactions were detected from 120 crosses (6.7 %) of bacteria isolated from a single dog. These results indicate that the prevalence of coaggregation between plaque bacteria is similar in the dog and the human; Gibbons and Nygaard (1970) reported coaggregation in 9 % of their crosses with human plaque bacteria.

Comparison to bacteria isolated from humans

Although coaggregation was observed at a similar frequency in dogs compared to previous studies using bacteria from humans, autoaggregation appeared to be more common in dogs. In the literature pertaining to oral bacteria from humans, autoaggregation is often not detected or not mentioned, so there is no need to correct for autoaggregation, and coaggregation may therefore be detected more easily. In the present study for example, seven isolates (25 %) were found to autoaggregate under standard conditions (Table 5.3), but in a similar study using 23 isolates of human origin no autoaggregation was detected (Gibbons and Nygaard, 1970). The higher level of autoaggregation in canine dental plaque may indicate an ecological difference compared to human dental plaque.

It seems probable that canine teeth experience greater hydrodynamic shear forces and are thus less able to sustain thick biofilms. In such a situation there may be greater competition for direct adhesion to the substratum, and autoaggregation could be an adaptation for this purpose. This speculation is supported by the work of Rickard *et al.* (2004), which showed that high hydrodynamic shear forces were associated with a high frequency of autoaggregation in freshwater biofilms, and coaggregation was more frequent under lower shear condtions. In the absence of coaggregation partners, autoaggregation may assist exit of bacteria from the bulk fluid and subsequently stabilize the biofilm structure in the same way as coaggregation; however, the opportunity for metabolic cooperation is reduced.

Direct comparison of coaggregating pairs with examples of human origin is difficult because the species found in canine dental plaque are not usually found in human dental plaque. Although there are many shared genera, streptococci and fu-

sobacteria are conspicuously rare in the dog and these are among the most prolific coaggregators in human dental plaque, along with *Actinomyces* species (Kolenbrander *et al.*, 1995). Several *Actinomyces* species were isolated from canine samples, and these were found to coaggregate with *Leptotrichia*, *Neisseria*, *Porphyromonas*, and *Streptococcus* species, consistent with their promiscuous coaggregation interactions in human dental plaque.

The *Fusobacterium* isolate used in the assay of a single dogs oral microbiota did not appear to have the near universal coaggregation behaviour that is often reported for *F. nucleatum* from humans, however autoaggregation may have masked this property if present. The *Haemophilus* species, haem1, would also have had any coaggregation reactions masked by its strong autoaggregation.

Porphyromonas species have been recognized as important bridging organisms in human dental plaque; by having multiple coaggregation partners, some species facilitate the association of other species which do not coaggregate directly with each other (Kolenbrander *et al.*, 1985). The results from this study suggest that this role also applies to *Porphyromonas* species in canine dental plaque, for example *Porphyromonas gulae* (bpp6) was found to coaggregate with *Granulicatella* sp., *Streptococcus* sp., *Neisseria* sp., *Staphylococcus epidermidis*, *Actinomyces bowdenii*, and *Fusobacterium* sp.

Canine-adapted assay conditions

The slightly higher rate of coaggregation observed under standard conditions compared to canine-adapted conditions may be due to the fact that not all possible crosses were tested in the former experiment.

The visual assay was chosen to assess coaggregation in preference to using a spectrophotometer because it is easier to perform, and Cisar *et al.* (1979) found that comparable results were obtained with both methods. Correction for autoaggregation had the undesirable effect of masking some interactions, but it avoided the possibility of declaring false-positive interactions. To adapt the standard coaggregation buffer for bacteria isolated from dogs, the pH was lowered to 7.5 and the salts composition was adjusted, according to measurements made at the Waltham Centre for Pet Nutrition. In addition, the pH buffer was changed to HEPES which is

more biologically compatible than TRIS (Good *et al.*, 1966), and the buffer strength was increased to 0.01 M because the pH of the standard buffer was observed to be unstable.

Transmission electron microscopy

TEM revealed dense fimbriae on haem1 which may be related to the strong autoaggregation observed with this bacterium. The exact identity of this isolate was not established and it seems likely that it represents a previously undescribed species of the *Haemophilus* genus, based upon its 16S rRNA gene sequence (Section 3.3.1) and its morphology presented here. The taxonomy of this genus is presently in dispute and it is frequently referred to as *Actinobacillus* (Potts *et al.*, 1985).

Actinobacillus actinomycetemcomitans is recognised as an opportunistic periodontal pathogen of humans, and is also known to be a highly adhesive organism (Henderson *et al.*, 2003). Non-fimbriated smooth colony variants of *A. actinomycetemcomitans* have a reduced ability to adhere to hydroxyapatite and saliva-coated hydroxyapatite (Rosan *et al.*, 1988), therefore the presence of fimbriae on this isolate may enable it to adhere to the tooth surface as a primary coloniser.

Coaggregates of lep1 with actino3, and fuso3 with cory2 were also clearly observed. The coat surrounding cory2 cells appears similar to the cell wall layering observed on corynebacteria by Puech *et al.* (2001), due to the unusual cell envelope structure of corynebacteria which contains an outer polysaccharide barrier layer similar in function to the outer membrane of Gram-negative bacteria.

Corynebacterium felinum was first isolated from a necrotic mouth lesion in a dead wild cat (Collins *et al.*, 2001), and later the *C. felinum*-like organism (cory2) used here was isolated from the dental plaque of three dogs (Chapter 3). Coaggregation of cory2 with a *Fusobacterium*-like species from a dog was detected by TEM but not by the coaggregation assay, highlighting the problem of autoaggregation masking coaggregation reactions.

The genera *Leptotrichia* and *Fusobacterium* are closely related (Conrads *et al.*, 2002) but were found to have different coaggregation partners. The coaggregation differences may be related to the structural differences observed by TEM, lep1 having a very plain and uniform cell surface but fuso3 having a complex undulating

appearance.

5.5 Conclusions

These experiments have shown that coaggregation occurs among the bacteria constituting the canine oral microbiota in a similar way to that observed many times with bacteria from human dental plaque. Although the species in these communities differ, genera common to both seem to exhibit similar coaggregation behaviour, in particular there is evidence that *Porphyromonas* species perform a bridging function and *Actinomyces* species also coaggregate prolifically in canine dental plaque, as they do in humans.

No universal coaggregators were detected which could play a role similar to *F. nucleatum* in human systems, but this may have been due to masking by autoaggregation. Autoaggregation was found to be more common in bacteria of canine origin compared to that reported in the literature regarding oral bacteria from humans, and this may indicate a selective advantage for primary colonisers rather than secondary colonisers in canine dental plaque.

Chapter 6

In vitro microcosm development

6.1 Introduction

An *in vitro* microcosm model of canine dental plaque was developed to allow experiments to be performed on plaque-like biofilms without using real dogs. Using a model has certain advantages over taking samples from animals, in particular the experimenter can have greater control over environmental parameters, and obtaining samples is generally easier and better controlled. Since its first application in oral microbiology by Wilson *et al.* (1995), the CDFF has been widely employed in the field, and has proved particularly useful for producing microcosm dental plaque biofilms (Pratten and Wilson, 1999; McBain *et al.*, 2003).

Other useful systems for the production of microcosm dental plaque include the Sorbarod perfusion system, the flow cell, and the modified Robbins device as discussed in Section 1.4, however the CDFF was considered to be the most useful model for the initial studies presented here. In particular, the operation of the CDFF provides an environment similar to the *in vivo* tooth not provided so well by the other alternatives. The rotating turntable and flow of medium simulates the action of saliva over dental enamel without permanently submerging the substratum, and a natural atmospheric equilibrium is allowed to develop.

A total of five independent CDFF experiments were performed, and these were named Canine1 - Canine5. Initial experiments, Canine1 and Canine2 are presented in this chapter. These experiments used a saliva inoculum and did not produce char-

acteristic oral biofilms, but provided a basis for model development. The conditions for the latter three experiments were adjusted accordingly, and these effectively form a triplicate repeat of the same conditions, presented in Chapter 7.

6.2 Materials and Methods

Initial CDFF experiments, Canine1 and Canine2, were based on the methods of Pratten and Wilson (1999) for the development of laboratory microcosms from human saliva. These were inoculated with two 1 ml vials of canine pooled saliva and fed with sterile CAS at a rate of 0.5 ml min^{-1}.

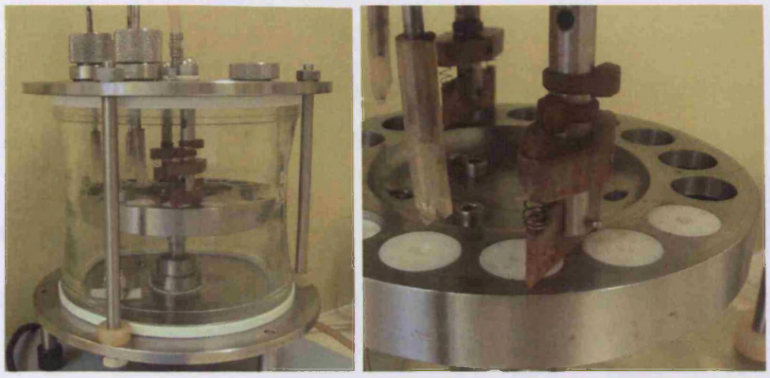
6.2.1 Constant depth film fermenter (CDFF)

The CDFF (supplied by University of Wales, Cardiff, UK) is shown in Figure 6.1. It consists of a rotating turntable, which holds fifteen PTFE pans located flush around its rim. The pans have recesses which hold the chosen substratum material, upon which the biofilms are allowed to develop. The medium flows onto the biofilms, and excess growth is removed by scraper blades which sit flush with the top of the pans, hence the biofilms are kept at a constant depth. The biofilm thickness is determined by the depth to which the substratum is recessed into the pan. After flowing over the rotating pans, the medium drips into the base of the CDFF and drains away. A base plate scraper blade was added to ensure efficient drainage and prevent blockages.

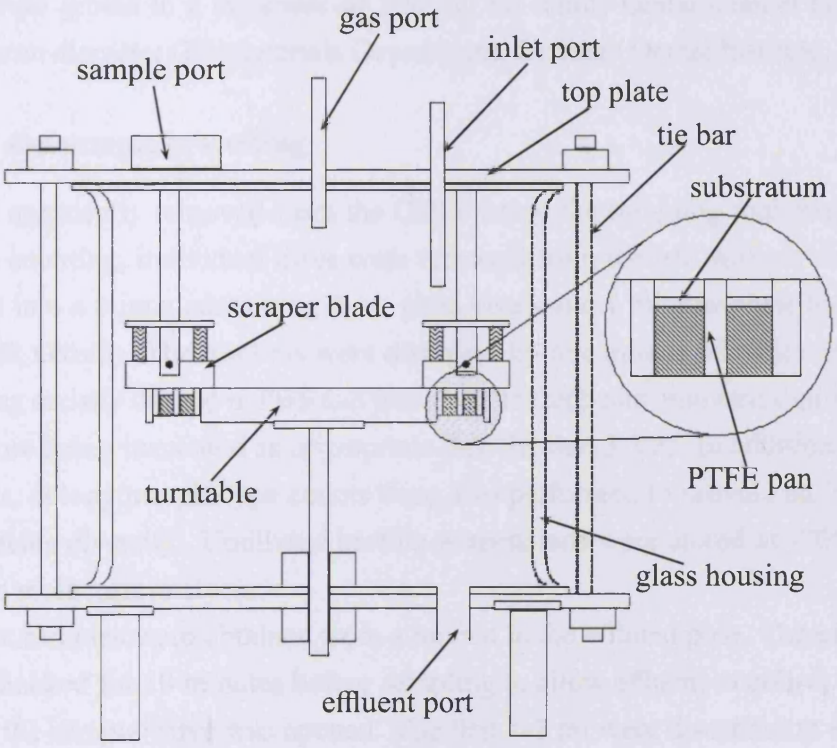
The biofilms, turntable, and associated apparatus are all housed within a sealed enclosure which contains an air space of approximately three litres. This space was vented to the outside atmosphere via a 0.2 μm filter, allowing the microcosm to exchange gases and develop a gaseous equilibrium with the air. It is also possible, if desired, to supply a defined gas mixture to the microcosms.

Inoculum

The inoculum consisted of 2 ml canine pooled saliva (CPS), collected and stored as described in Section 2.3.



(a) CDFF. (b) CDFF turntable.



(c) CDFF schematic.

Figure 6.1: Constant depth film fermenter. A stainless steel and glass housing contain a stainless steel turntable, into which PTFE pans are set. The pans contain the growth substratum recessed to a pre-determined depth. Growth medium flows in through an inlet port on the top plate, onto the rotating turntable. Fixed scraper blades ensure even distribution of growth medium to all pans, and also serve to limit the thickness of biofilms growing on the substratum.

Inoculation

The inoculum was added to 500 ml CAS maintained at 37°C, which was continuously mixed and pumped into the CDFF over a period of eight hours.

Growth medium

All experiments used canine artificial saliva (CAS) (Section 2.5.2) as the growth medium.

Substratum

Biofilms were grown to a thickness of 200 μm on canine dental enamel cut into discs of 2 mm diameter (Biomaterials Department, Eastman Dental Institute, UK).

Sampling and sample processing

Pans were aseptically removed from the CDFF using the sampling tool provided. For viable counting, individual discs were removed from the pan without rinsing, and placed into a bijoux containing three glass beads and 1 ml phosphate buffered saline (PBS, Oxoid). The biofilms were disrupted by one minute of vortex-mixing before being serially diluted in PBS and plated out in duplicate onto agar-containing media before being incubated as appropriate (see Section 3.2.2). In addition to viable counts, colony morphotype counts were also performed to provide an indication of species diversity. Undiluted biofilm suspensions were stored at -70°C for subsequent molecular analyses.

Effluent samples were obtained from a branch in the effluent pipe. The effluent pipe was blocked for 10 minutes before sampling to allow effluent to collect in the pipe, then the sample valve was opened. The first 1-2 ml were discarded to ensure stagnant effluent was not included in the sample. Effluent samples were used for viable counts, microscopic examination, molecular analyses, and pH determination (BDH Gelplas combination electrode).

Viable counts were determines as described in Section 3.2.2, and are expressed per mm^2 substratum which equates to a volume of 6.3×10^{-4} ml in the case of a biofilm grown to a depth of 200 μm. Selective growth media were used to indicate

the numbers of specific bacterial groups in some experiments; the results from these counts were checked by Gram-staining and comparative 16S rRNA gene sequencing (Section 3.2.3).

6.2.2 Serum addition experiment

Initial CDFF experiments produced biofilms of low species diversity, so the growth medium was modified by addition of horse serum to simulate the gingival crevicular fluid (GCF), which must enrich the environment at or near the gingival sulcus *in vivo*. It has been shown previously that the distribution of major proteins in GCF is similar to that found in serum (Estreicher *et al.*, 2004). The effect of serum supplementation was assessed by a simple experiment as follows.

CAS was prepared and dispensed into duplicate universal bottles and made up to a final volume of 19 ml with sterile horse serum constituting 0%, 20%, 40%, or 60% of the total volume (after addition of 1 ml inoculum). To mimic the inoculation conditions used in the CDFF, a standard inoculum was prepared by mixing 1 ml pooled canine saliva and 1 ml pooled canine plaque into 23 ml standard CAS.

CAS broths were pre-warmed and inoculated with 1 ml standard inoculum before being incubated statically overnight at 37°C. After 18 hours and 42 hours one of each broth pairs was vortex-mixed, diluted in PBS, and plated out onto CBA and AA for viable counting. The standard inoculum was also plated out in the same fashion. After processing, the undiluted broths were examined microscopically as wet preparations and after Gram-staining. An assessment of the biodiversity of each broth was made by counting the different morphotypes that developed on the viable counting plates, and using these results to generate biodiversity indices for each broth. The biodiversity measures used were the Shannon-Weaver index, H (Equation 6.2; Shannon and Weaver, 1963), calculated from the proportions of each species in the sample, Pi (Equation 6.1); and the total number of morphotypes - termed 'richness'.

$$Pi = \frac{number\ of\ a\ given\ species}{total\ number\ of\ organisms} \tag{6.1}$$

$$H = -\sum_{i=1}^{i} Pi.\ln(Pi) \tag{6.2}$$

6.3 Results

6.3.1 Cultural analyses

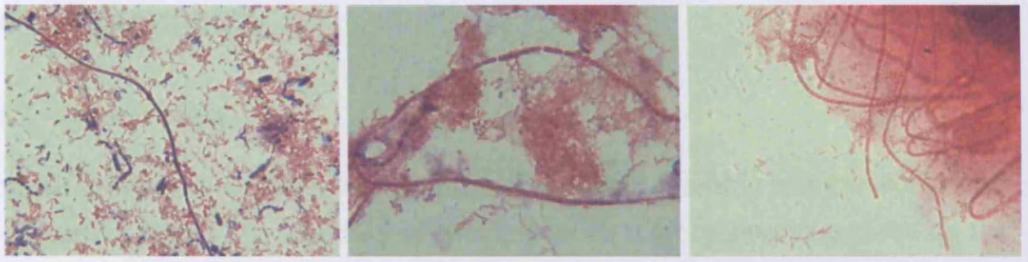
Non-selective growth media revealed a low species richness in initial CDFF experiments, as indicated by colony morphologies, and additional counts using selective media supported this result. Each morphotype that grew on the selective media was individually counted and sub-cultured, and then a partial 16S rRNA gene sequence from each type was used for identification. The sequence-based identities showed that the biofilms were composed of a Gram-negative anaerobe (not sequenced), *Streptococcus* species, *Escherichia coli*, *Enterococcus* species, and *Pseudomonas aeruginosa*. Sequencing of the predominant morphotypes recovered from Canine1 revealed that these biofilms were dominated by *Pseudomonas aeruginosa*.

6.3.2 Serum addition

The value of adding serum to the growth medium (CAS) for modelling of canine dental plaque was assessed by inoculating broths containing from 0 - 60 % serum, and examining the resulting bacterial consortia. Microscopic examinations of wet preparations (not shown) and Gram-stains (Figure 6.2) from the broths after 18 hours and 42 hours of incubation revealed a diverse range of bacteria in all broths regardless of the serum concentration.

Species diversity was perceived to be greater in the broths containing 0 % to 20 % serum compared to the broths containing a higher proportion of serum when they were observed microscopically. This was particularly noticeable in the wet preparations which revealed a vast array of motile bacteria, including spirochaetes (see Figure 6.2b for Gram-stained examples) in the broths of low serum content and much fewer motile bacteria in the broths containing 40 - 60 % serum. Greater species diversity at lower serum concentrations was also apparent from the Gram-stains, which revealed a selection for Gram-negative species at high serum concentrations.

A measure of species diversity was made for each broth by assuming the viable count for each colonial morphotype represents a different species. This allowed a number of measures and indices relating to biodiversity to be calculated, including the Shannon-Weaver index. The Shannon-Weaver index of diversity averaged 1.4



(a) 0 % (b) 20 % (c) 60 %

Figure 6.2: Gram-stains showing 42 hour old CAS broths inoculated with canine plaque and saliva, and supplemented with 0 %, 20 %, or 60 % horse serum.

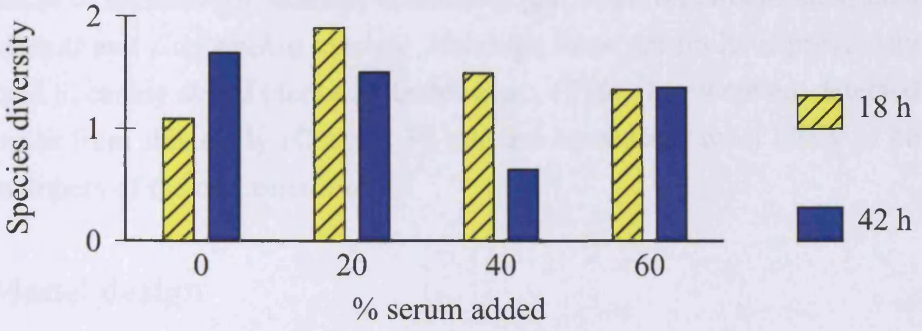


Figure 6.3: Effect of serum addition to CAS on species diversity (Shannon-Weaver) in static broths inoculated with canine plaque and saliva. Hatched=18h, plain=42h.

with maximum and minimum of 1.9 (18 hours, 20 % serum) and 0.6 (42 hours, 40 % serum) respectively (Figure 6.3). The most morphotypes (richness measure) isolated from a single broth was 13 different types from the broth containing no added serum after 42 hours growth; this broth also had the second highest Shannon-Weaver index. The evenness measure indicates how similar the relative numbers of different morphotypes are, in this case the most even distribution of morphotypes were found in the 20 % serum broth after 18 hours, and the 0 % serum broth after 42 hours growth.

6.4 Discussion

6.4.1 Overview

Biofilms grown in the CDFF were examined by viable counting using selective and non-selective agars. The identities of the predominant morphotypes counted on the selective agars were confirmed by Gram-stain and, in some cases, by 16S rRNA gene sequencing. This revealed that, in general, the selective agars were ineffective.

Biofilms from initial experiments were characterised by low species diversity and dominance of bacteria not normally considered part of the oral microbiota, such as *Pseudomonas* and *Escherichia* species. Although these genera have previously been detected in canine dental plaque (Wunder *et al.*, 1976), they were not detected in the samples from this study (Chapter 3), and are considered most likely to be transient members of the oral microbiota.

6.4.2 Model design

Growth medium and inoculum

The poor performance of the initial CDFF experiments in respect to modelling canine dental plaque biofilms can most easily be explained by a deficiency in either the inoculum or the growth medium, or both. The results from the CAS serum supplementation experiment revealed a likely inadequacy in the pooled saliva inoculum used for initial CDFF experiments, and this was supported by data presented in Chapter 3 which showed that the saliva did not contain many of the genera found in plaque samples.

The CAS serum experiment used a combined inoculum of pooled saliva and pooled dental plaque, and even with no serum added a rich consortium of characteristic oral bacteria developed in CAS broths, indicating that the complex medium is able to support plaque-like microcosms. Despite this, it seemed clear that the model could be improved by incorporating a GCF substitute, since GCF has a profound influence upon microbial communities in the mouth.

Although not part of the saliva which comes from salivary glands, the GCF is a serum-like exudate which emerges from the gingival crevice, carrying with it an

array of growth substrates and inhibitory factors which should not be ignored in oral biofilm models. Inflammation of the gingival margin due to gingivitis is accompanied by an increase in plaque accumulation, probably due to the increase in GCF flow rate which accompanies gingivitis (Hillam and Hull, 1977; Rüdiger *et al.*, 2002). Rüdiger *et al.* (2002) showed that increased GCF flow rates result in increased levels of plasma proteins in the pellicle, and in turn the levels of these proteins could be linked to the prevalence of certain bacteria in the plaque, highlighting the importance of GCF in plaque models. They found that *Streptococcus* species were generally inhibited by the presence of plasma proteins, but the prevalence of *Actinomyces* spp., *Prevotella* spp., and *Fusobacterium nucleatum* were all positively correlated with plasma protein levels in the pellicle.

The use of biodiversity indices to compare bacterial consortia and biofilms is considered ideal for assessing dental plaque models since dental plaque is a diverse microbial consortium, so the selection of the model parameters producing greatest biodiversity can be reasonably assumed to represent the most appropriate set up. The measures of biodiversity made in the CAS serum experiment were based upon the colonial morphotypes recovered from each broth upon serial dilution and inoculation onto non-selective blood agar plates. This is clearly a rather crude method for quantifying the numbers of different bacteria present in the broths and probably always underestimated the true biodiversity of the sample, however if the counting efficiency remains constant then a fair comparison can be made. The purpose of this experiment was to determine whether serum supplementation would be beneficial to the development of *in vitro* canine dental plaque in the CDFF.

Since the results for all of the broths were rather similar and have not been replicated, the small differences between them are unlikely to be statistically significant. Although the biodiversity comparison does not demonstrate a clear benefit, it seemed reasonable that supplementing the CAS with 5 % horse serum during CDFF experiments is unlikely to have a detrimental effect, but is likely to improve the nutritional qualities of the medium.

In healthy humans, the GCF flow rate has been reported to be approximately 1 % of the saliva flow rate (Smith, 1992), however it emerges in direct contact with the teeth but saliva is released from glands distal from the teeth. Supplementation at a rate of 5 % was therefore expected to represent a biologically relevant concentration

by compensating for these spatial factors, although ideally analyses of plaque fluid in dogs should be made to check this.

Serum supplementation may encourage the persistence of certain fastidious bacteria such as spirochaetes, and its inclusion in this laboratory model of canine dental plaque serves to mimic the GCF.

Other possible modifications and developments

Other workers have supplemented AS for use in a CDFF plaque model in a defined way with 0.1 g l^{-1} cysteine hydrochloride, 0.001 g l^{-1} haemin, and 0.0002 g l^{-1} vitamin K_1 (McBain *et al.*, 2003), presumably to encourage the persistence of fastidious organisms such as *F. nucleatum*, *P. gingivalis*, and spirochaetes etc. They also included in their model an artificial food medium which was pulsed into the CDFF periodically to simulate mealtimes, and this had a measurable impact upon the community composition.

Due to the link between GCF flow rate and periodontal disease status, modulation of serum flow rates in the CDFF could be used to simulate different disease states in oral models, perhaps incorporating the addition of blood too, however this was outside the scope of the present work. The use of simulated meals and diurnal cycles (e.g. in salivary and GCF flow rates) may further improve the model by more accurately modelling the natural system. Ecologically speaking, the benefit of such cycles is that partucular organisms are not allowed to become dominant simply because their physiology matches the uniform environment provided. Instead, by varying the environmental conditions, advantage is given to different organisms at different times, thus encouraging the development of more diverse and stable biofilms.

6.5 Conclusions

CDFF experiments based on the methods described by Pratten and Wilson (1999) for producing microcosms from human dental plaque were modified by use of a canine-adapted artificial saliva, but did not produce microcosms characteristic of dental plaque. Biofilms were formed, but they were characterised by low species

diversity and were dominated by species which are not indigenous to the oral cavity of humans or dogs.

Although oral microcosms are often generated from human saliva, the saliva inoculum used in this study was thought to be the main deficiency in the system, preventing plaque-like biofilms from developing, so it was decided to add plaque to the inoculum for future experiments. An experiment was performed to see if the addition of serum to the medium might also help by modelling GCF, although again successful plaque-like microcosms have been generated from human samples without considering this factor (e.g. Pratten *et al.*, 1998). The serum supplementation experiment indicated that large amounts of serum might reduce the bacterial diversity, and did not show a clear benefit over an experiment of 42 hours, however it was decided to include serum at a lower concentration than used in the experiment, at a level similar to that expected *in vivo*.

The experience and modifications developed during these initial experiments formed the basis of further experiments which are described in Chapter 7.

Chapter 7

Validation of *in vitro* microcosm

7.1 Introduction

In Chapter 6, the human dental plaque microcosm system described by Pratten and Wilson (1999) was modified for the *in vitro* modelling of canine dental plaque. The modifications included use of canine artificial saliva as the growth medium, canine dental enamel as the substratum, and canine saliva as the inoculum; however the resulting microcosms were not similar to canine or human dental plaque.

Based upon this finding and further experimetal work, two additional modifications were made to improve the system as a model of canine dental plaque; the inoculum was supplemented with canine dental plaque, and the growth medium was supplemented with horse serum to simulate GCF. Experiments repeated in triplicate using these conditions are the subject of this chapter. Owing to sampling limitations and gradually acquired experience, some of the analyses performed on these experiments varied slightly between replicates as detailed in the relevant sections.

7.2 Materials and Methods

7.2.1 Constant depth film fermenter (CDFF)

Inoculum

The inoculum consisted of 1 ml canine pooled saliva (CPS) and 1 ml canine pooled plaque (CPP), collected and stored as described in Section 2.3.

Inoculation

Please refer to the method described in Section 6.2.1.

Growth medium

All experiments used canine artificial saliva (CAS) (Section 2.5.2) as the growth medium, and this was supplemented with sterile horse serum (Oxoid) at 5 % v/v. CAS is a modification of the complete artificial saliva described by Pratten *et al.* (1998) for modelling human dental plaque in the CDFF.

Substratum

Please refer to the method described in Section 6.2.1.

Sampling and sample processing

Please refer to the method described in Section 6.2.1.

7.2.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out as described in Section 4.2.2, using primers 357F-gc and 518R. Template DNA was prepared from laboratory microcosms by using the DNA extraction method described in Section 2.7.

Banding pattern analyses

Digital gel images were scaled, aligned, and merged using ImageJ (Rasband, 2005) with the marker lanes as a guide. The plot profile function of ImageJ was then used to extract pixel intensity data from each gel lane, then the exact pixel location of each band was identified by correlating visible bands with increases in pixel intensity. Each band was identified by the distance in pixels it had migrated down the gel, and the presence or absence of each band was recorded in binary for each lane.

Binary lane profiles were used to generate neighbour joining trees using the unweighted pair group method with arithmetic means (UPGMA), to investigate relationships between profiles. The computer program PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1999) was used to generate UPGMA distance matrices, which were repeated 1000 times using subsets of the data for bootstrap analysis. The Consense program of the Phylip software package (Felsenstein, 1993) was used to generate consensus trees indicating the most probable true topology based upon the 1000 input trees.

7.2.3 PCR detection of culture-resistant bacteria

DNA extractions from biofilm and effluent samples of Canine4 were used as a template for selective amplification of 16S rRNA gene sequences of *Spirochaetes* and TM7 group bacteria as described in Section 4.2.3.

7.3 Results

7.3.1 Cultural analyses

Canine3

Viable counting on selective agars combined with confirmation by 16S rRNA gene sequencing on experimental run Canine3 confirmed the presence of a diverse microbiota composed of typical oral genera, and also revealed species succession as would be expected from natural dental plaque (Figure 7.1). Sequencing of distinct

morphotypes from each agar clearly showed that, in most cases, the selective agars used did not work as intended so further use of these was halted (Chapter 6).

For the first two days Gram-positive cocci were the most numerous bacteria in the biofilms at approximately 1×10^6 cfu mm^{-2}, with *E. coli* present in similar numbers and obligately anaerobic species typically being detected at approximately 1×10^5 cfu mm^{-2}. By eight days into the experiment, the number of Gram-positive cocci had reached a plateau of 5×10^6 cfu mm^{-2}, and the number of *Porphyromonas* species reached a plateau of 6×10^7cfu mm^{-2}. *Fusobacterium* species reached 1×10^7 cfu mm^{-2} after 16 days, becoming more numerous at this point than the Gram-positive cocci, and second only to the *Porphyromonas* species count. *Actinomyces* species were first detected after 8 days and persisted until 16 days at approximately 1×10^6 cfu mm-2. Similarly *Pasteurella* species were detected at approximately 1×10^6 cfu mm^{-2} after 16 and 24 days.

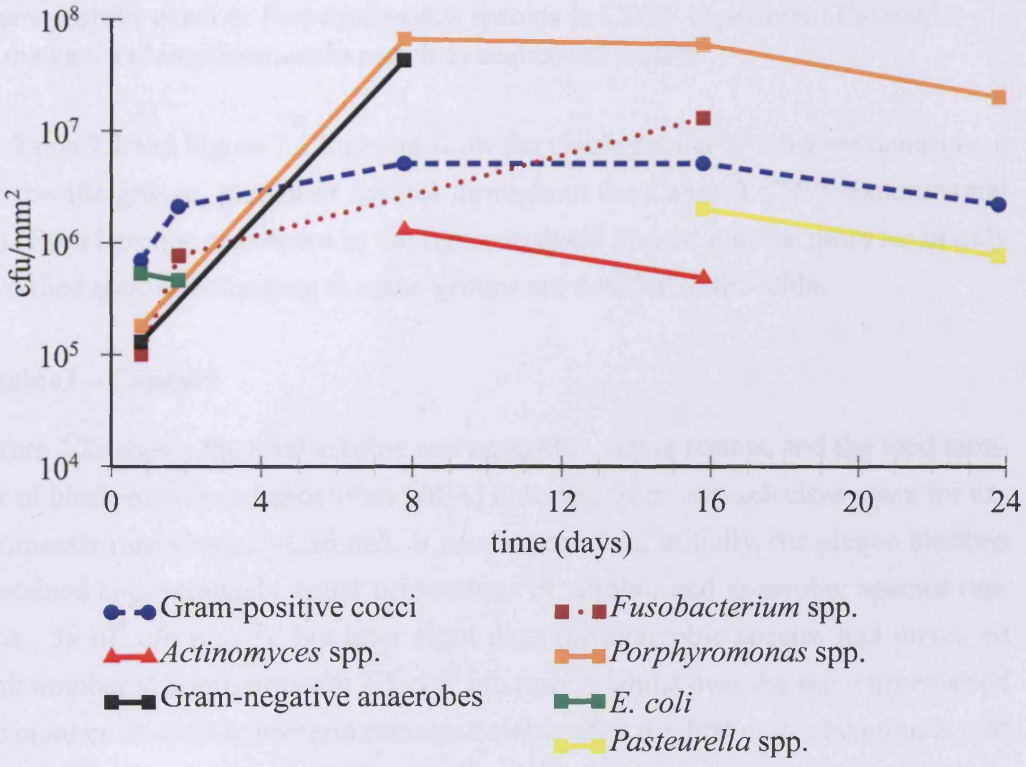


Figure 7.1: Viable counts for specific taxa determined for Canine3 CDFF experiment using selective agars, Gram-stain, and 16S rRNA gene sequencing for confirmation.

The data shown for Canine3 in Figure 7.1 are from duplicate counts on single biofilms because the other biofilms were allocated for microscopic and molecular analyses (Section 7.3.2 and Section 7.3.3). As such, these data should be regarded only as a crude indicator of the community in Canine3 because the magnitude of errors could not be evaluated.

Group	Proportion / ID	method of colony identification
Gram positive cocci	49 % *Streptococcus* spp.	1 by sequence, 4 by cg match
	39 % unknown	(11 unidentified types)
	8 % *Streptococcus suis*	1 by sequence, 1 by cg match
	4 % *Enterococcus* spp.	1 by sequence, 1 by cg match
Porphyromonas species	53 %*Porphyromonas catoniae*	1 by sequence, 2 by cg match
	47 % unknown	(3 unidentified types)

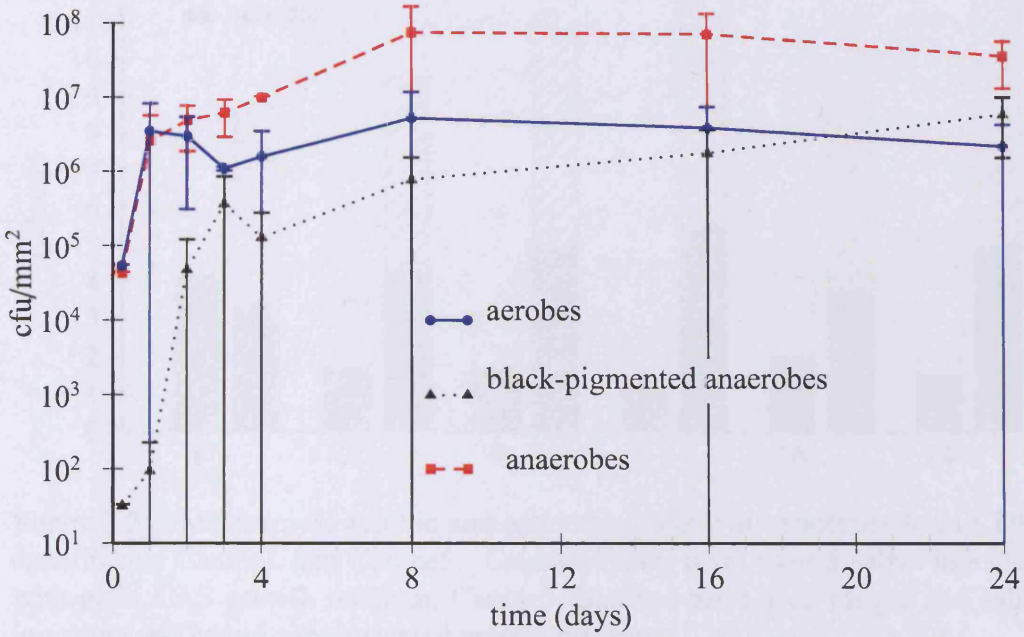
Table 7.1: Breakdown of genus species identifications from colonies identified as Gram-positive cocci or *Porphyromonas* species in CDFF experiment Canine3. cg match = colony/Gram-stain match to sequenced isolate

Table 7.1 and Figure 7.1 together show the viable counts which were determined for specific groups, genera or species throughout the Canine3 CDFF experimental run. Broad groups are shown in the figure to avoid clutter, and the more accurately identified species belonging to some groups are detailed in the table.

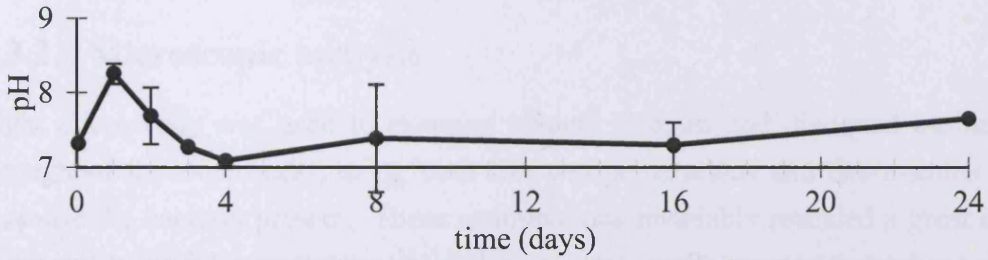
Canine3 – Canine5

Figure 7.2a shows the total aerobic and anaerobic viable counts, and the total number of black-pigmented anaerobes (BPA) obtained from non-selective agars for experimental runs Canine3-Canine5. It can be seen that, initially, the plaque biofilms contained approximately equal proportions of aerobic and anaerobic species (approx. 5×10^4 cfu mm^{-2}), but after eight days the anaerobic species had increased their number to approximately 7.5×10^7 cfu mm^{-2}, whilst over the same time period the number of aerobic bacteria remained stable after the first day. The number and proportion of black-pigmented anaerobes in the biofilms increased continuously throughout the experimental runs, initially comprising just 0.03 % of the cfu but after 24 days comprising 15.7 % of the cfu. The pH of the effluent remained stable

between 7.0 and 8.0 throughout the experiments except for a peak of 8.3 at day 1 (Figure 7.2b).



(a) Mean viable counts, error bars indicate standard deviation of the mean (n=3).



(b) Effluent pH (experiments 3 and 5), error bars indicate standard deviation of the mean (n=2). If no error bar is shown then the measurement is from experiment 5 only.

Figure 7.2: Mean viable counts from biofilms, and effluent pH during CDFF experiments Canine3 - Canine5.

Canine1 and Canine5 species richness

Species richness as determined by the number of different colony types arising from each sample for experiments Canine1 and Canine5 are shown in Figure 7.3. This figure allows comparison of the initial and modified experimental conditions. It can be seen that the richness was always greater in experiment Canine5, which used a plaque enriched inoculum, and serum enriched growth medium.

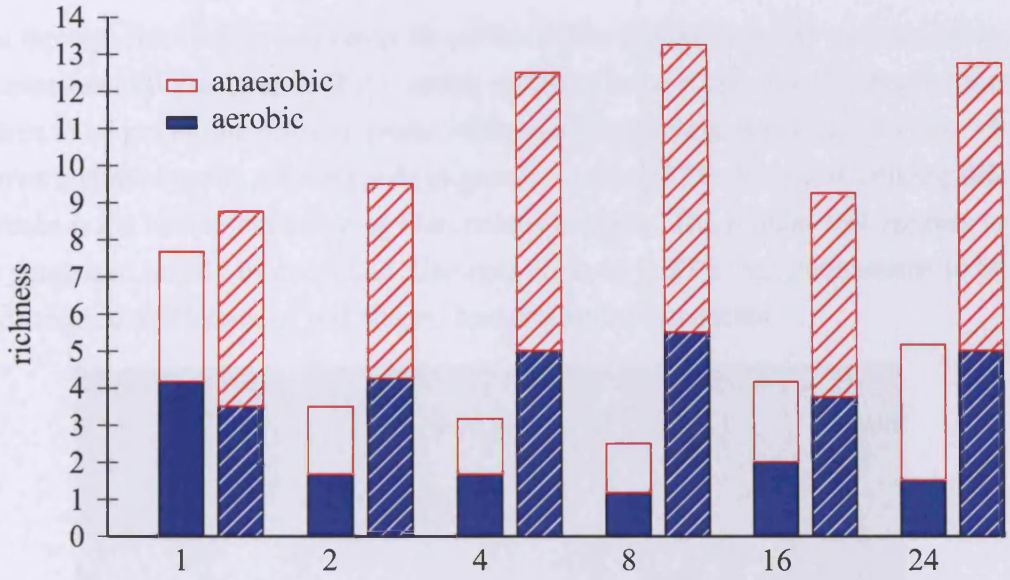


Figure 7.3: Mean (n=4) aerobic and anaerobic colony morphotypes from CDFF experiments Canine1 and Canine5. Canine1 (plain bars) used a saliva inoculum with plain CAS growth medium, Canine5 (hatched bars) used plaque and saliva inoculum and serum supplemented growth medium.

7.3.2 Microscopic analysis

Light microscopy was used to examine effluent samples and disrupted biofilms throughout the experiment, using both live wet preparations and Gram-stains to visualise the bacteria present. These examinations invariably revealed a great diversity of bacterial morphotypes including several motile species throughout the duration of the experiments.

Scanning electron microscopy (SEM) of biofilms removed from the CDFF revealed a biofilm structure characteristic of dental plaque (e.g. Sukontapatipark *et al.*, 2001). Figure 7.4 shows the edge of a biofilm stack after 48 hours growth in the CDFF, and a large section of the substratum can also be seen. The substratum is largely colonised by cocci which may be *Streptococcus*, *Gemella*, *Neisseria*, or *Granulicatella* species among others. Cocci and small rods can be seen forming into microcolonies, and there is an extensive matrix of filamentous bacteria covering the substratum. These filamentous bacteria appear to be adherent to other bacteria on the substratum, especially to the microcolonies, and it can be seen that the filaments

run through the biofilm and cover its surface. The biofilm is partly encased in an extracellular polysaccharide (EPS) which obscures some of the cells, but the biofilm seems to be predominantly composed of filamentous bacteria and cocci. Figure 7.5 shows a similar scene after eight days growth in the CDFF. The most striking difference is the lower abundance of filamentous bacteria. The biofilm still appears to be composed largely of cocci and filaments encased in EPS, but there seems to be an increased abundance of rod-shaped bacteria and spirochaetes.

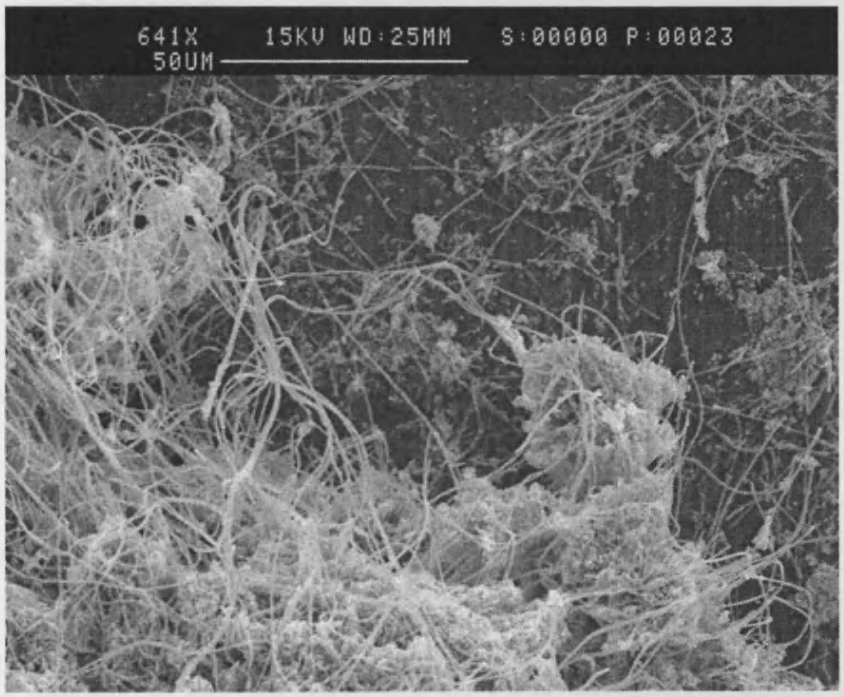


Figure 7.4: SEM of 48 hour old biofilm from experiment Canine3 showing the edge of a biofilm and exposed substratum.

Confocal laser scanning microscopy revealed similar features to those seen by SEM, but the use of nucleic acid stains allowed specific visualisation of cells, leaving extracellular material unstained. A time series of z-projections from experiment Canine5 is presented in Figure 7.7.

The confocal images, although taken from a different CDFF experimental run, show a similar succession and ultrastructure to that observed by SEM. One and four day old biofilms appear to have an open structure with large spaces spanned by conspicuous filamentous bacteria which appear in places to be attached to other

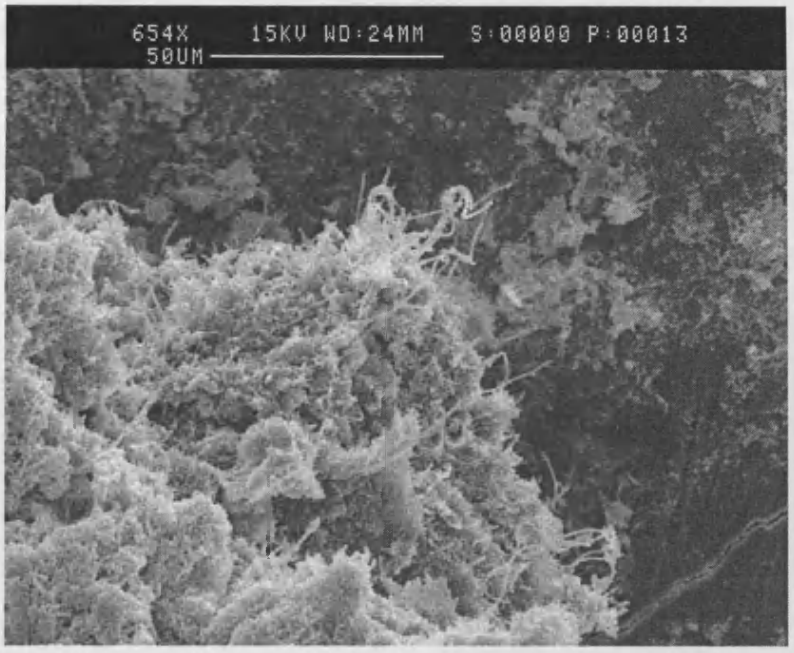


Figure 7.5: SEM of 8 day old biofilm from experiment Canine3 showing EPS encased biofilm next to exposed substratum.

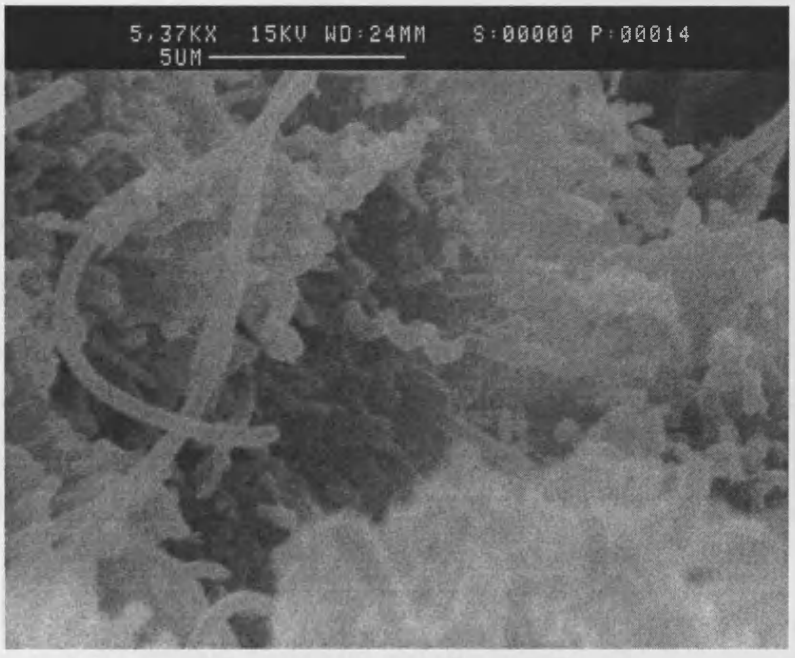
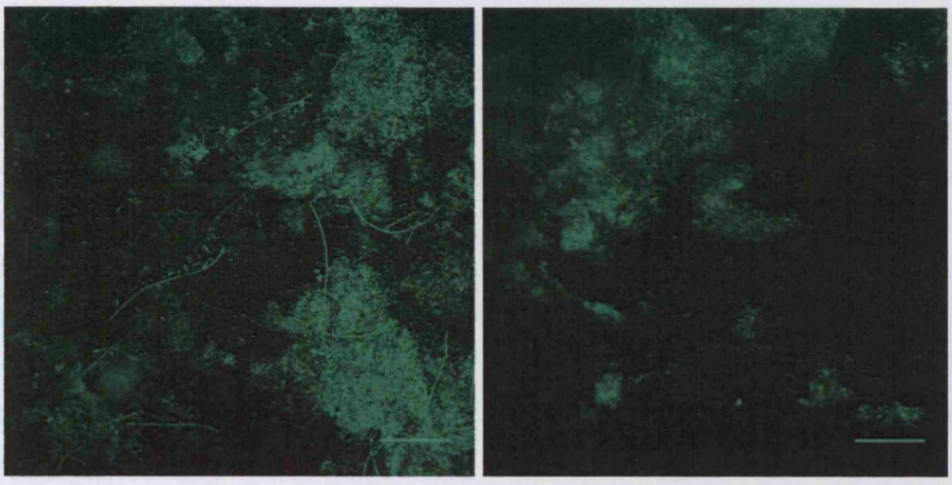
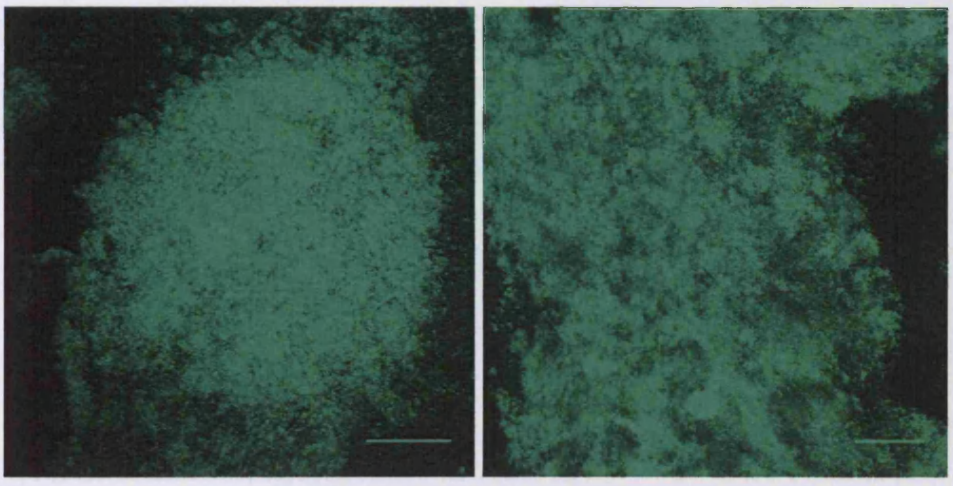


Figure 7.6: High power SEM of 8 day old biofilm from experiment Canine3 showing EPS encased biofilm with *Spirochaetes*.

bacteria, either singly or in clumps. Unlike the SEM images, it can be seen from the confocal images that the filaments are composed of individual cells of variable length. Measurements of 50 filaments from 7 images ranged from 4 μm to 26 μm, with cells on average measuring 12 μm long. The image from a 16 day old biofilm shows a single large colony approximately 130 μm across composed predominantly of cocci and short rods, and the 24 day biofilm seems to have a similar composition.



(a) 1 day. (b) 4 days.



(c) 16 days. (d) 24 days.

Figure 7.7: Series of CLSM images showing biofilm development in the CDFF, from experiment Canine5. Scale bars indicate 30 μm.

The z-projections presented here show maximum pixel intensity data for each

pixel position and this obscures much of the volume information of the scan, particularly on the more mature biofilms in which most pixel positions are occupied by cells at some point in the volume. Examination of individual layers from the scans showed that despite the relatively uniform appearance in the 16 day and 24 day z-projections, the biofilms were in fact composed of many different cell types, including the filaments clearly visible on the earlier images.

7.3.3 Community profiling (DGGE)

DGGE profiles were obtained for CDFF experiments Canine3 and Canine4, using DNA extracted from biofilms for both experiments, and also using DNA from the effluent of experiment 4. The effluent profile was obtained to determine whether a comparable profile to the biofilm would be obtained, or whether owing to the greater biomass available, a more rich profile may be obtained. Careful visual observation combined with pixel intensity measurements were used to determine the presence or absence of bands at defined migration distances to allow comparison of profiles between different samples.

It can be clearly seen in Figure 7.8 and Figure 7.9 that banding patterns change through the course of a CDFF experiment. These figures also show the number of distinct bands detected for each sample, giving an indication of the ecological richness which can be seen to peak at 2 days for both biofilm profiles (14 or 15 bands). The richness peak for canine4 effluent was at 8 days (19 bands).

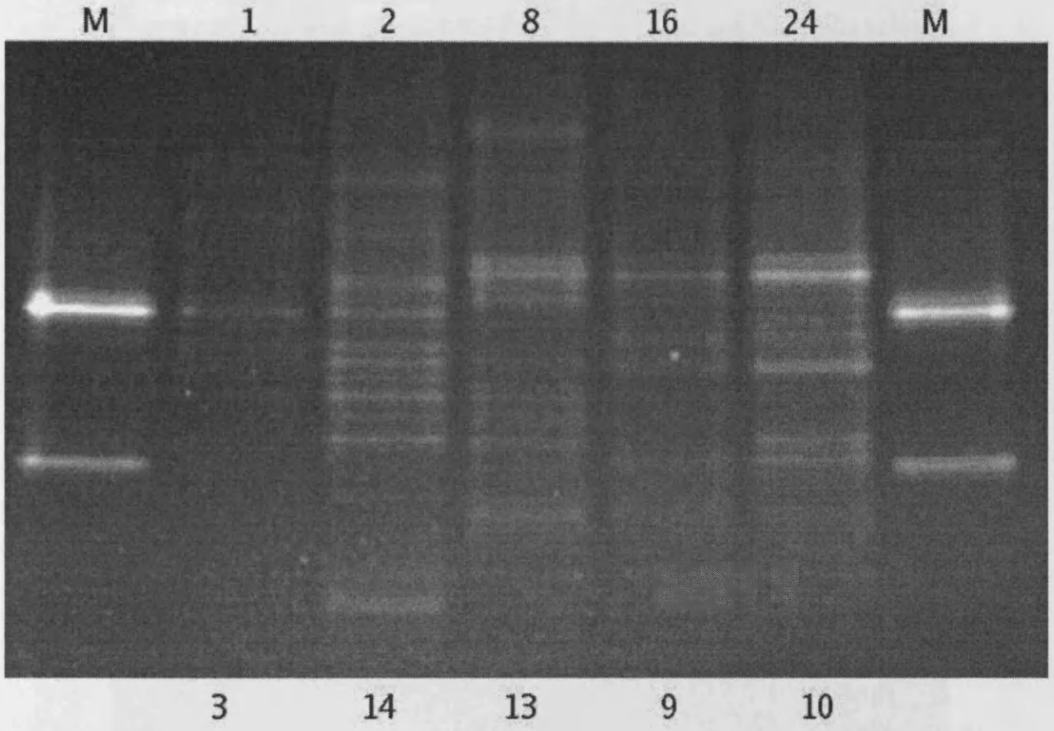
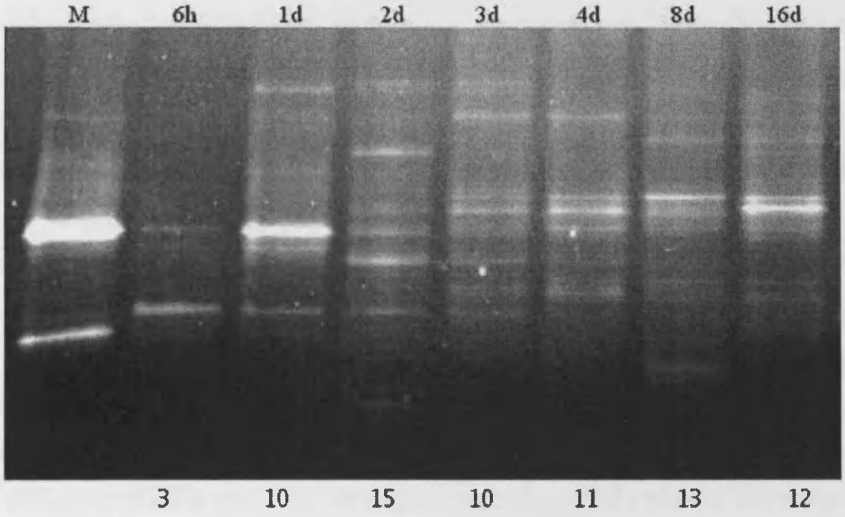
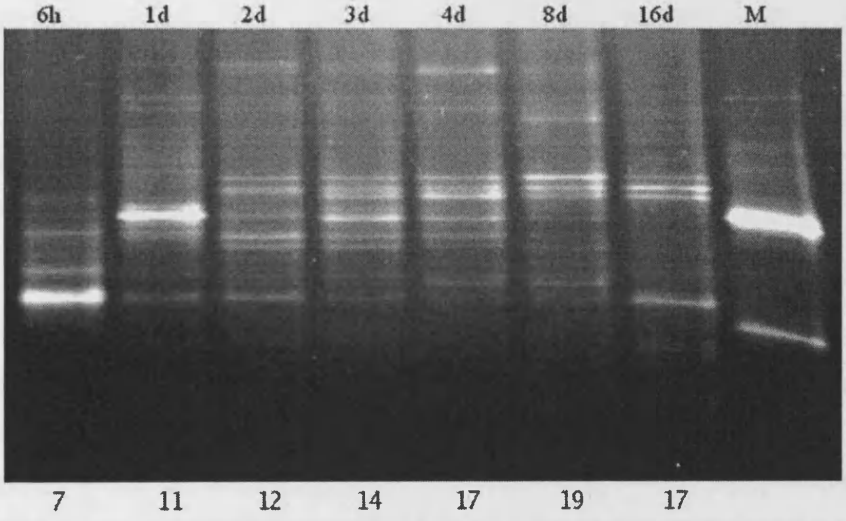


Figure 7.8: Denaturing Gradient Gel Electrophoresis profile for biofilm samples taken from CDFF experiment Canine3. Numbers above lanes indicate age of sample in days, M indicates marker lane containing amplicons from *Tannerella forsythensis* (top) and *Prevotella intermedia*, numbers below lanes indicate the number of bands detected.



(a) biofilm.



(b) effluent.

Figure 7.9: Denaturing gradient gel electrophoresis profiles for biofilm and effluent samples taken from CDFF experimental run Canine4. The two images are both from the same physical gel, which had marker lanes (M) at each end containing amplicons from *Tannerella forsythensis* (top) and *Prevotella intermedia*. Numbers above lanes indicate the age of sample in days or hours, numbers below lanes indicate the number of bands detected.

Visual interpretation of DGGE profiles is difficult beyond noting presence or absence of certain bands between samples which soon becomes impractical. To help overcome this, the banding patterns were converted into a binary sequence indicating presence or absence of each defined band, and this was used to cluster the patterns and display them as a tree (Figure 7.10).

Bootstrapping of the tree indicates the reliability of the topology, but branch lengths are unitary and carry no information. In 100 % of cases, the 6 h biofilm from canine4 formed the basal node of the tree, and this was closely linked with 6 h and 1d effluent samples, and the 1d sample from canine3; from the gel images it can be seen that these are also among the simplest patterns obtained. Near the middle of the experiments, linkages become less clear but, in general, samples clustered depending upon which experiment they were from; i.e. Canine3 biofilm, Canine4 biofilm, or Canine4 effluent. The strongest linked pair of profiles were the last two Canine3 samples taken at 16 and 24 days (99.5 %).

Apart from the basal 6 h node, all of the Canine4 biofilm profiles clustered together, with the 1-4 day samples forming one distinct clade, and the 8 and 16 day samples forming another. The Canine3 profiles also clustered together in a similar way, except for the 1 day sample which clustered with canine4 6 h, and the 2 day sample which clustered with more mature samples from Canine4.

7.3.4 Culture-resistant bacteria

The presence of spirochaetes and TM7 group bacteria in biofilms was indicated by products of the predicted size after selective PCR from CDFF (Canine4) effluent samples but not biofilm samples (Figure 7.11 and Figure 7.11). The possibility that non-target taxa were amplified was checked by cloning and sequencing a selection of the PCR products according to the methods described in Section 2.13.

Cloning

Cloning of PCR products was attempted for positive TM7 bands corresponding to CDFF effluent samples from 1 day and 16 days, as described in Section 4.2.3.

The gel image in Figure 7.12 shows the first five M13 clone PCR products for the two TM7 positive bands selected, and it can be seen that products of the correct

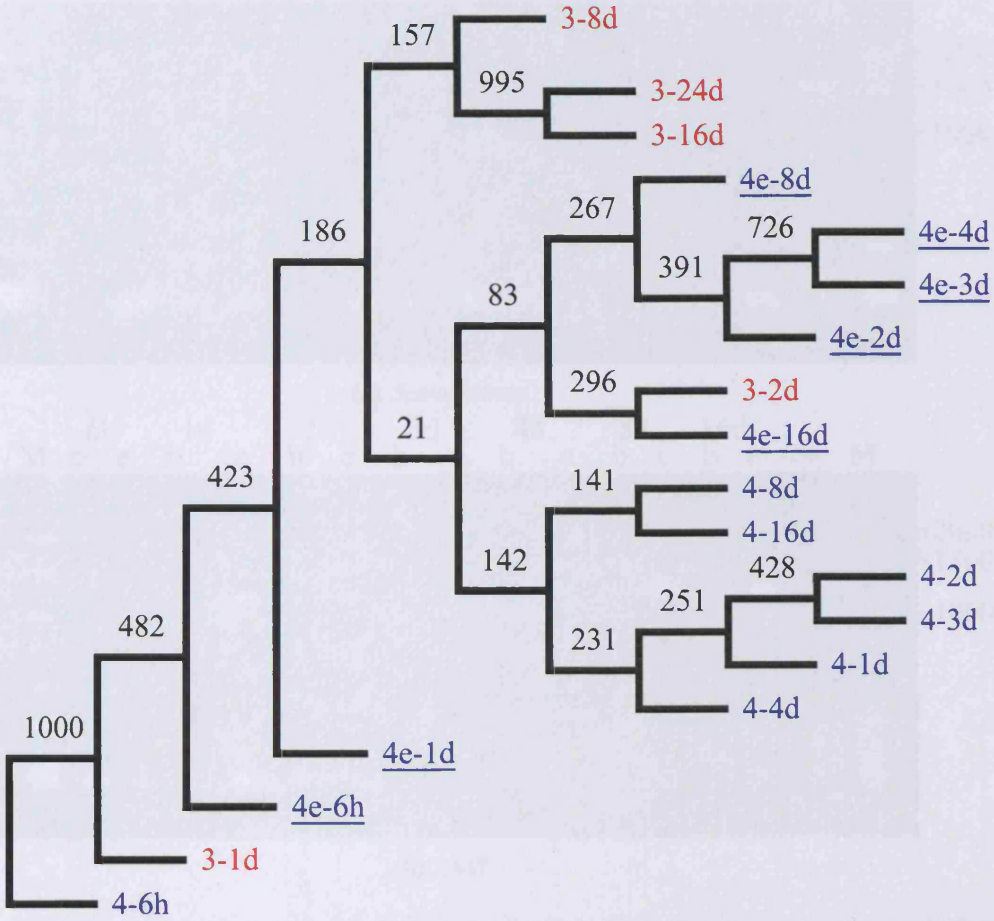
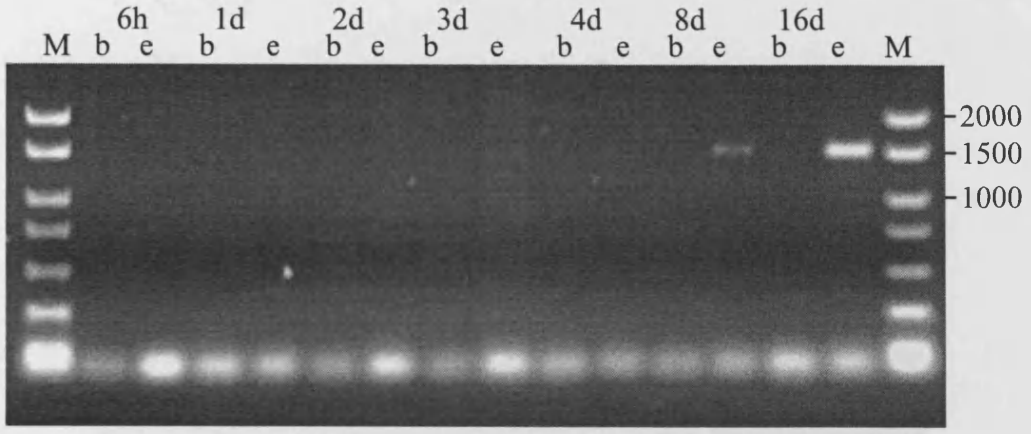


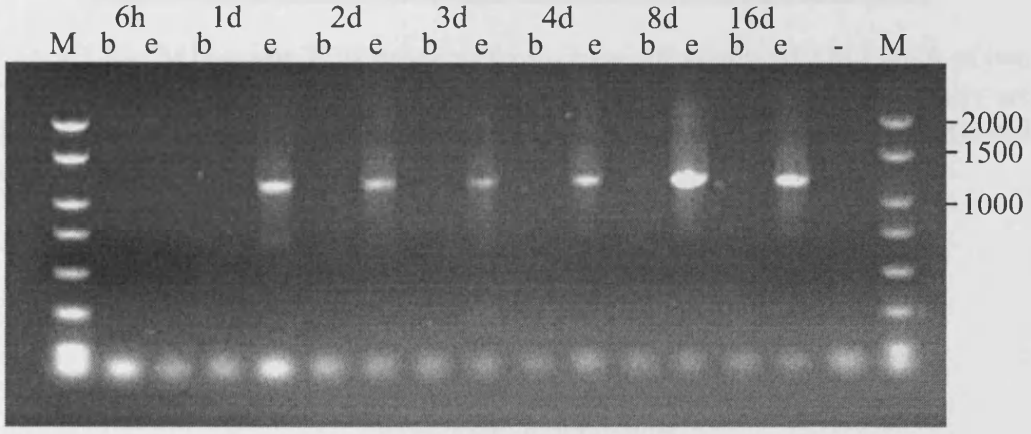
Figure 7.10: Consensus bootstrapped UPGMA tree based on binary band data from CDFF DGGE profiles, from experimental runs Canine3 and Canine4. Numbers at nodes indicate the number of times each particular branch was formed out of 1000 replicate trees. Samples are labelled in the form 'experiment-time point', and profiles generated from effluent samples are underlined.

size (approximately 1200 bp) were obtained for both cloned PCR products; a total of 20 correct inserts were detected from the 40 clones tested. Five clones for each of the positive bands were sequenced and the identity of the originating organism was determined by comparison to the sequences available on GenBank using BLAST.

Only one clone was positively identified as TM7 and this was from the 1 day effluent sample (Table 7.2), the sequence of this clone has been deposited in GenBank with accession number DQ156985. The other clones from the 1 day sample were



(a) *Spirochaetes.*



(b) TM7.

Figure 7.11: Gels showing products of selective PCR for *Spirochaetes* and TM7 bacteria from experiment Canine4 in CDFF effluent (e) and CDFF biofilm (b) DNA extractions. Relevant band sizes (bp) are labelled for the marker lanes (M).

all identified as *Neisseria* species, and four different species were identified from the 16 day sample. Three of the clones from the 16 day sample were identified as organisms which have been previously identified from canine dental plaque according to the GenBank records; *Bacteroides denticanoris* (2 clones) and *Wernerella denticanis* (1 clone).

The tree in Figure 7.13 shows the TM7 clone sequence relationship to other TM7 sequences from GenBank which were identified as significant matches by a BLAST search. The source of each sequence according to the GenBank record is shown in brackets, and it can be seen that the closest match was to a sequence from

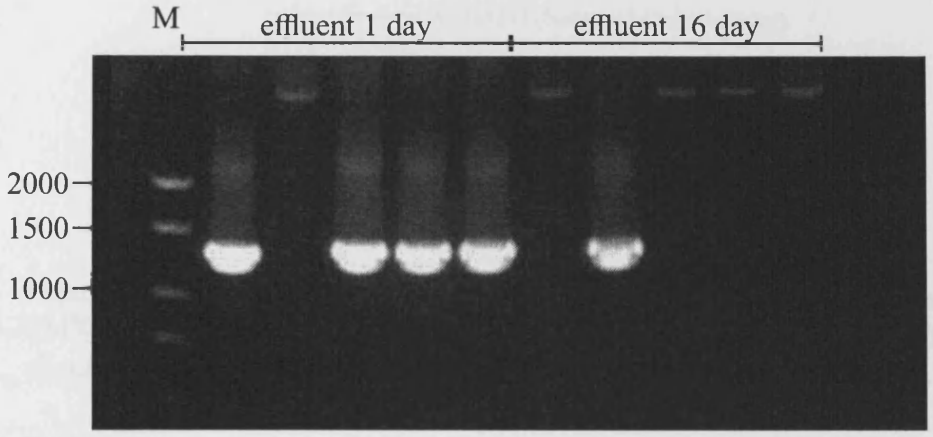


Figure 7.12: M13 insert PCR products from clones of amplified TM7 DNA of two CDFF effluent samples. Five clones from effluent samples at 1 day and 16 days are shown, along with a size marker (M) for which relevant band sizes are indicated (bp).

days	Identification/match
1	1× TM7 phylum sp. oral clone
	3× *Neisseria weaveri*
	1× *Neisseria animalis*
16	2× *Bacteroides denticanoris*
	1× *Wernerella denticanis*
	1× *Aquamicrobium defluvium*
	1× *Aneurinibacillus migulanus*

Table 7.2: BLAST based identifications for sequences of TM7 PCR-clones from CDFF effluent. The sequences used to make these identifications are available on GenBank with accession numbers DQ156985-DQ156994.

human subgingival plaque. The sequence identity between these two sequences was found to be 95.7 %.

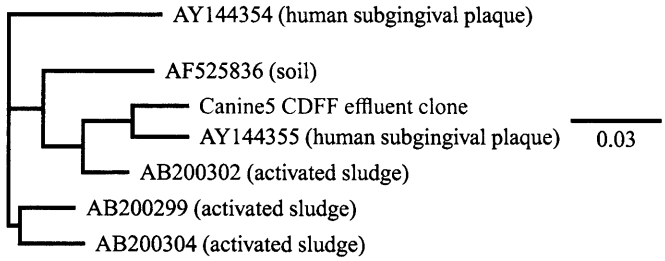


Figure 7.13: Phylogenetic tree showing the relationship between candidate division TM7 bacteria from GenBank and a PCR-clone sequence from CDFF effluent (897 alignment positions).

7.4 Discussion

7.4.1 Overview

Biofilms grown in the CDFF were examined by light and electron microscopy, and by viable counting using selective and non-selective agars. The identities of the predominant morphotypes counted on the selective agars were confirmed by Gram-stain and in some cases 16S rRNA gene sequencing.

Biofilms from initial experiments (Chapter 6) were characterised by low species diversity and dominance of bacteria not normally considered part of the oral microbiota, such as *Pseudomonas* and *Escherichia* species. Although these genera have previously been detected in canine dental plaque (Wunder *et al.*, 1976), they have not been detected in dental plaque from this study (Chapter 3), and are considered most likely to be transient members of the oral microbiota. Later experiments using a modified inoculum and growth medium supplemented with horse serum produced microcosms similar in community and physical structure to dental plaque.

7.4.2 Model validation

Cultural analyses

Initial experiments were easily identified as poor models because simple consortia of non-oral bacteria developed. For the later experiments presented in this chapter, cultural measures indicated that successful plaque-like microcosms derived from

canine plaque and saliva had been established. This was evidenced by the characteristic species succession from aerobic Gram-positive species towards anaerobic Gram-negative species (Figure 7.1), not to mention the smell.

In addition, the *E.coli* detected at the beginning of Canine3, presumably a contaminant in the inoculum, appears to have been quickly out-competed by more characteristic plaque bacteria, indicating that the experimental system is providing an environment similar to the tooth *in vivo*, and which therefore favours typical plaque bacteria. The detection of anaerobic genera such as *Porphyromonas* throughout the experimental runs shows that, despite operating the CDFF under aerobic conditions, the model is providing a niche for strictly anaerobic bacteria, presumably due to bacterial activity and metabolic co-operation in the biofilm. Using atmospheric gases is considered preferable to artificially reducing the oxygen tension as it allows the biofilm to develop in a more natural fashion, although it may be worth considering elevating the carbon dioxide tension to model the influence of respiration.

Microscopic analyses

It is significant that light and electron microscopy detected spirochaetes living in the laboratory biofilms as these bacteria have very strict growth requirements and most of them have never been cultured in the laboratory (Chan and McLaughlin, 2000). It seems quite likely that these bacteria are able to persist in the CDFF because they are benefiting from the activities of other bacteria in the biofilms.

Spirochaetes are widely considered to be important members of dental plaque and are strongly associated with periodontal diseases, being reported as rare or undetected in healthy sites, and comprising 50 % or more of the microscopic count in plaque removed from diseased sites (Loesche, 1988). Despite this, spirochaetes are often ignored in studies of plaque bacteria owing to their elusive nature.

Confocal and electron microscopy revealed biofilm structures similar to those which have been previously observed in dental plaque and CDFF plaque microcosms (Sukontapatipark *et al.*, 2001; Hope and Wilson, 2003), with many bacterial morphologies present throughout the experiments. The most conspicuous cells in many images were long filaments composed of cells 4 - 24 μm long, averaging 12 μm. These cells appear very similar to the as-yet uncultured TM7 cells identified in

human subgingival plaque using FISH by Brinig *et al.* (2003), which were reported to be 4 - 30 μm long, averaging 12 μm. The morphological similarity is strong evidence that TM7 cells persisted in the CDFF for 24 days, and this could be confirmed by using FISH on biofilms from the CDFF.

If TM7 cells can be confirmed to be growing in the CDFF then this would give further evidence that the system is generating plaque-like microcosms, and would provide an excellent opportunity for investigating this recently discovered and so-far uncultured bacterial division.

DGGE

The utility of DGGE for fingerprinting saliva-derived microcosms grown in the CDFF has been shown previously (McBain *et al.*, 2003), however the analysis of DGGE banding patterns remains something of an enigma. The excision and sequencing of selected bands is often used to identify some of the organisms in a community (e.g McBain *et al.*, 2003) and this approach certainly has some value, especially when investigating samples of poorly characterised or simple environments. Unfortunately, the short sequences obtained (typically < 200 bp), combined with the problem of co-migration severely limit the usefulness of this approach.

With complex communities, co-migration means that bands need to be purified before sequencing, and this can be achieved by PCR-cloning of the band into a suitable vector, and clones selected for subsequent sequencing. Due to the considerable labour and expense required for this endeavour, alternative strategies such as community PCR-cloning and sequencing are likely to prove more favourable if phylogenetic data are required from a complex community.

In this study, some phylogenetic data were obtained by sequencing of cultured isolates from the CDFF, and by performing selective PCR reactions targeted at spirochaete and TM7 16S rRNA genes. DGGE was used to provide an indication of species richness throughout CDFF runs, and provide a profile to allow comparison of samples at the community level.

Species richness was assessed by counting bands for each profile using the assumption that one band equates one operational taxonomic unit. This indicated that the model is able to maintain a rich consortium, for example after 16 days an av-

erage of 13 bands were detected. There is certainly not a one-to-one relationship between bands and taxa in a sample, but for the purposes of model validation it is a reasonable assumption given that comigration and multiple bands from single taxa may cancel each other out, and since some taxa will be less amenable to the PCR amplification step or present at low concentrations, the assumption probably under estimates the true species richness of the sample. This can be clearly seen by increasing richness with respect to time in the CDFF which is a closed system; bands are probably not produced at the early time points because there is insufficient biomass. It would also be possible to use DGGE profiles to estimate diversity by assuming that band intensity indicates species abundance, however this is unlikely to be the case due to a number of factors such as PCR bias and variable 16S rRNA gene copy number per cell.

The clustering analysis of DGGE banding patterns may have been expected to produce one of two general outcomes; patterns should cluster by time point, or by experiment. Clustering by time point would indicate a high level of reproducibility between experiments, but this was generally not observed except at early time points when the patterns were all very simple. Once communities became established, or had sufficient biomass, the DGGE patterns clustered by experiment, indicating a high level of community stability within experiments but not between experiments.

It was hypothesized that the effluent and biofilm patterns from the same experiment (Canine4) may be similar enough that regular effluent sampling could be used as an alternative to sacrificing valuable biofilms, and that the greater biomass available in the effluent may improve the sensitivity of DGGE.

Whilst slightly more bands were detected in effluent samples, the clustering analysis showed that the effluent and biofilm communities were quite different. The nature of this difference may warrant further investigation; the difference suggests that certain species are being selectively shed from the biofilm, effectively into the saliva. This is unlikely to be an advantageous situation for the shed organisms which would probably be swallowed *in vivo*, so the implication is that intense competition in oral biofilms has led to the evolution of biofilm re-modelling strategies including active expulsion of competitor organisms from the biofilm. If this is the case, then such mechanisms could be exploited for therapeutic use, and careful monitoring of biofilm and effluent DGGE profiles from CDFF microcosms would be a sensible

starting point for such investigations.

Culture-resistant bacteria

Selective PCR reactions indicated the presence of *Spirochaetes* and TM7 bacteria throughout CDFF experimental run Canine4, but these indications were not verified except in one case. Two bands from the TM7 PCR, corresponding to 1 day and 16 day effluent samples, were cloned and sequenced, showing that in both cases the bands were produced due to amplification of more than one taxon. This was expected since direct sequencing of bands generated mixed output, highlighting the requirement for band purification by cloning.

A TM7 sequence was verified from the CDFF effluent one day into the experiment and this was closely related to a sequence previously detected in human subgingival plaque (Paster *et al.*, 2001). Failure to verify TM7 sequences from the other tested sample indicates that these bacteria may be lost from the system and that the detection after 1 day could be due to TM7 presence in the inoculum, not necessarily indicating persistence in the CDFF. The detection of four different taxa from this latter (16 day) sample also indicates that the PCR conditions used are not very specific for the target division. To determine conclusively whether TM7 bacteria are able to persist in the CDFF it would be necessary to sequence a larger number of clones, and preferably from a number of time points, or FISH could be used.

Sequencing of clones from the 16 day effluent sample showed that two of the phylotypes present were first described from samples of canine dental plaque, showing that the system is able to support these plaque related species for at least 16 days.

7.5 Conclusions

These experiments have shown that plaque-like microcosms can be grown in the CDFF from a canine plaque and saliva inoculum, using an artificial saliva supplemented with horse serum as the growth medium, and canine dental enamel as the substratum.

Characterisation of biofilms by culture using selective agars was found to be

unreliable because the agars, especially CFAT, failed to select for the specified bacteria. The typical succession of plaque bacteria elucidated from the data was not obtained simply by virtue of the selective media. Instead, the main function of the selective media in this study was to provide a range of distinguishable colony types which could be counted and later identified by Gram-stain and sequencing. The MS agar was found to be quite successful in selecting for *Streptococcus* species and could probably be relied upon to give a rough estimate of these bacteria without further confirmation.

CFAT agar was found to be unreliable for isolating *Actinomyces* species, as has previously been found by McBain *et al.* (2003), who identified all of the distinct colony morphotypes growing on CFAT from artificial human plaque grown in the CDFF, and found that none of them were *Actinomyces* species. In light of this, the CFAT chemicals were also tried at triple strength, but to no avail.

As with human dental plaque, *Streptococcus* species were found to be dominant during the initial colonisation of the canine dental enamel, although the actual species involved were different to those found in humans. Once the *Streptococcus* species had colonised the substratum in this experimental run, their numbers remained relatively constant whilst the other bacteria increased to approximately 10 times their number, however they could still be detected by the use of MS agar. It is likely that without using MS agar (as in experimental runs Canine1 - Canine3) the *Streptococcus* species would have seemed to disappear from the system as the plaque matured.

Molecular methods showed that diverse bacterial communities were sustained in the CDFF for up to 24 days when the experiments were halted. Although most of the bacteria in the microcosms were not identified to species level, the combination of culture-based data, microcsopic data, and DNA sequences showed that the biofilms were composed of bacteria similar to those found in human dental plaque, and in some cases identical to those found by culture in canine dental plaque as part of this study (Chapter 3).

A candidate division TM7 sequence was obtained from a 1 day CDFF effluent sample, and this confirmed that bacteria from this group were present in the inoculum. Microscopic analyses indicated that the as-yet-uncultured TM7 group bacteria persisted in the CDFF throughout the 24 day duration of experiments, and

spirochaetes were also occasionally detected by SEM and light microscopy. Detection of these culture-resistant indigenous oral bacteria in the CDFF is taken to be a strong indication that the model is generating an environment and microcosm similar to that found *in vivo*, since the exacting growth requirements of these organisms are very difficult to replicate in the laboratory in isolation.

Chapter 8

Conclusions

The oral microbiota of dogs has, not surprisingly, been under-represented in the literature compared to the human oral microbiota. In general, previous studies have been carried out from a human perspective, either as a model of human oral conditions or to investigate the organisms which may be transferred to humans by biting. Few of these studies have considered 16S rRNA gene based taxonomy when identifying bacteria, partly because many such studies were performed before the necessary tools were available; instead the canine oral microbiota has therefore been forced to conform to the taxonomy of a human-centred scheme, resulting in frequent mis-classification of organisms from dogs as similar organisms known to occur in human mouths.

Modern sequencing technology has given taxonomy the power to break free of arbitrary classifications based upon human convenience, and has revealed the flaws and successes of the old methods. This is shown by the findings of this study on the canine oral microbiota which has relied heavily on public sequence databases for the identification of bacteria. Of 84 bacterial phylotypes isolated from the canine oral cavity, approximately half represented novel species, probably often not because these organisms have never been seen before, but because they have been identified as similar organisms previously found in humans.

It is quite remarkable that only 28 % of the isolates obtained in this study were identified as being indigenous to humans, given that human dental plaque is among the most intensively studied biofilm systems and that humans and dogs are often in

close contact. The clearest conclusion to draw from this is that humans and dogs have their own distinct oral microbiotas despite superficial similarities. It is most probable that similar investigations performed on other animals would find also that these have their own distinct plaque consortia, and it would be interesting to find out if this is the case.

It is possible that a portion of the species found in humans and dogs represent oral generalists which may be found in a large number of hosts, or it may be that close contact between humans and dogs has resulted in some organisms adapted for one host occasionally surviving in the other when conditions permit. Such a situation would be more likely to arise if the microbiota of one host became displaced, for example by antibiotic therapy, thus allowing less well adapted organisms from the other host to colonise.

The evolutionary divergence of oral bacteria in the two hosts indicated by comparative 16S rRNA gene sequencing was surprisingly large, and this aspect may warrant further study. Comparisons with the oral microbiotas of other animals, especially those not kept as pets would complement this work well and help to clear up some of the evolutionary and ecological questions raised here.

Some of the bacteria isolated from the canine oral microbiota, including the entire recovered plaque community from one dog, were used in coaggregation studies based upon those pioneered in the 1970's for studies on the human oral microbiota (e.g. Gibbons and Nygaard, 1970; Cisar *et al.*, 1979). Identifying pairs or complexes of coaggregating bacteria provides a useful starting point for elucidating the complex structural and metabolic relationships which define dental plaque.

In this work, it has been shown that coaggregation occurs in bacteria from the mouths of dogs to a similar extent as in humans, but it was noted that the strength of interactions tended to be lower, and this could be attributed to a selective advantage for primary colonisers due to higher fluid shear forces in the canine mouth. The results indicated that certain genera seem to play similar roles in the canine mouth as their counterparts in the human, for example *Porphyromonas* species of both hosts are likely to perform a bridging function by bringing otherwise non-coaggregating pairs together, and *Actinomyces* species were found to be prolific coaggregators in the dog as they are in the human.

The coaggregation study could be followed-up by an identification of the pri-

mary colonisers of canine dental plaque, and this may be investigated by two complementary approaches. Canine teeth could be sampled with a swab at short intervals after cleaning; perhaps 10 minutes to an hour, to identify a selection of candidate primary colonisers. These could then be used in an adhesion assay using either canine dental enamel and a clarified saliva pellicle, or purified candidate pellicle receptors such as salivary amylase.

The CDFF has been used previously to model human dental plaque in the laboratory, allowing biotic and abiotic factors to be adjusted, and allowing downstream analyses of replicate biofilms. This model was adapted for growing microcosms resembling canine dental plaque using a plaque and saliva inoculum and a canine dental enamel substratum. Salts and pH of the growth medium described by Pratten and Wilson (1999) were adjusted according to measurements made on canine saliva at the Waltham Centre for Pet Nutrition, and the medium was supplemented with horse serum to mimic GCF.

It is interesting that supplementation of the saliva inoculum with plaque was required, because similar studies using a human saliva inoculum have been found to produce plaque-like biofilms. This may indicate that the saliva inoculum was compromised in some way, or that the bacterial load of canine saliva is lower than that of humans. The latter explanation seems feasible because dogs seem to salivate more than humans, therefore a dilution effect may occur, alternatively the anatomy and behaviour of the dog may be such that it tends not to suspend plaque bacteria into the saliva.

Culture-independent analyses of canine dental plaque were hampered by DNA extraction or PCR difficulties which may have been related to small sample size. The application of DGGE to the community fingerprinting of canine dental plaque was shown to have potential, and selective PCR reactions indicated the presence of TM7 bacteria and spirochaetes, but these results were not confirmed. The same methods were applied to laboratory microcosms with greater success; DGGE generated profiles from CDFF biofilm and effluent samples, and spirochaetes and TM7 bacteria were detected by PCR from the same samples. Again, the PCR amplicons were not confirmed by sequence, except for one TM7 sequence from a CDFF effluent sample.

Microscopy of CDFF-grown biofilms supported the selective PCR results, show-

ing that spirochaetes were present throughout CDFF runs by their distinctive morphology and motility. In addition, filamentous bacteria clearly visualised by SEM and CLSM were found to be morphologically similar to candidate division TM7 division bacteria previously observed in human subgingival plaque (Brinig *et al.*, 2003).

This work has shown conclusively that canines have a distinct oral microbiota compared to that of humans but, as expected, there are many similarities and shared species found in both hosts. The dog may however be a special case compared to other animals in this respect due to it's long friendship with humans. Many organisms found in one host may have an alter-ego in the other, but differences at the genus level indicate that the two microbiotas are fundamentally different and that a direct one-to-one similarity is unlikely. This interpretation was supported by the coaggregation study which showed that autoaggregation is a common phenomenon in dogs despite being very rare in humans.

Failure to produce plaque-like microcosms using a saliva inoculum also indicated a fundamental ecological difference between human and canine oral cavities. Plaque-like biofilms were, however, successfully produced using the CDFF with a plaque and saliva inoculum combined with a growth medium simulating canine saliva and GCF.

In conclusion, the present study has considerably advanced our knowledge of the constituent species of the canine oral microbiota, showed that they participate in coaggregation interactions, and demonstrated a method for the production of plaque-like microcosms from the same, which can provide a sound basis for further research on the improvement of canine oral healthcare.

Appendix A

Isolates data

Phylotype	Consensus ID	%	Closest BLAST	%	bp
abio1	**New species**	**NA**	***Granulicatella balaenopterae***	**91.2**	**621**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.18 Ø	colourless raised	-	diplococci - kidney	-	nd
cp08.23* Ø	brown pulvinate	+	rod	-	nd
cp10.19 Ø	brown convex, beta haemolytic	-	rod long, bulges in cell wall	weak	nd
cps01.04 Ø	small white dome	-	cocci	+	nd
cps01.06 Ø	colourless disc	+	cocci	-	nd
cps01.09 Ø	yellow dome	+	cocci clumps	+	nd
cps01.10 Ø	grey disc	+	cocci chains	-	nd
actino1	***Actinomyces canis***	**96.7**	***Actinomyces* sp.**	**96.7**	**643**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.02	white convex	-	rod	+	-
cp01.15 Ø	yellow convex	+	rod	+	nd
cp02.04	brown convex	+	rod	-	-
cp05.05	cream convex	+	rod	-	-
cp05.06	pale yellow convex	+	rod	-	-
cp05.14 Ø	white irregular umbonate	-	rod	+	nd
cp06.14 Ø	white convex	+	cocci chains, short	+	nd
cp06.25	white convex	+	rod	+	-
cp09.21 Ø	yellow convex, frilly edge	+	rod short	+	nd
cp10.07	small grey convex	-	rod	-	-
cp10.15 Ø	grey/cream convex	+	cocci	+	nd
cp17.01*	grey convex	+	rods, branching chains	-	-
cp17.03	yellow shiny convex	+	rod, branching chains	+	-
cps01.08 Ø	grey/yellow dome	+	cocco-bacilli chains	+	nd
cps01.12 Ø	small Yellow pimple	-	rod long	+	nd
cps01.18 Ø	yellow convex	nd	nd	nd	nd
actino10	***Actinomyces hyovaginalis* - like**	**98.9**	***Actinomyces suimastitidis***	**98.9**	**369**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.15 Ø	white pulvinate	-	rod	-	nd
cp05.21	brown raised	+	rod	-	-
cp05.27* Ø	white convex	-	rod	-	nd

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
cp10.16 Ø	pink convex	+	cocci small	+	nd
actino2	***Actinomyces canis***	**99.4**	***Actinomyces*** sp.	**99.4**	**697**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp06.18* Ø	cream convex	nd	nd	+	nd
cp06.23 Ø	yellow pulvinate	nd	nd	+	nd
cp17.06	shiny yellow pulvinate	-	rod, short chains	-	-
cps01.19	yellow dome	-	rod	+	-
actino3	***Actinomyces bowdenii***	**99.7**	***Actinomyces bowdenii***	**99.7**	**697**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp06.16 Ø	white convex	nd	nd	+	nd
cp07.01	white shiny convex	+	rod	+	-
cp07.13* Ø	yellow opaque convex	nd	nd	+	nd
cp08.15 Ø	yellow convex	+	rod short	+	nd
cp10.06	white convex	+	rod small	+	-
cps01.21	white convex	+	cocco bacilli	+	-
actino4	***Actinomyces bovis* - like**	**98.8**	***Actinomyces slackii***	**98.8**	**358**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.02	grey convex	+	cocco-bacilli	+	-
cp07.17 Ø	yellow irregular	-	rod irregular	+	nd
cp07.18* Ø	grey/yellow convex	nd	nd	+	nd
cp08.08	white convex	+	rod short, clumps	+	-
actino5	***Actinomyces hordeovulneris***	**98.4**	***Actinomyces hordeovulneris***	**98.4**	**396**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.04	white convex	+	rod	-	-
cp06.02*	white convex	-	rod long	-	-
cp07.02	white/yellow pulvinate	+	rod	+	-
cp08.13	white pulvinate	+	rod	+	-
cp08.22 Ø	white fuzzy irregular	+	rod	+	nd
cps01.24	light brown convex	-	cocco-bacilli	+	-
cps01.45 Ø	small umbilicate brown irregular	nd	nd	nd	nd
actino6	**New species**	**NA**	***Actinomyces hordeovulneris***	**94.9**	**658**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.03	colourless convex	-	rod	+	-
cp01.13 Ø	grey convex	-	rod	+	nd
cp07.05*	small colourless convex	-	rod	+	-
cps01.03 Ø	white dome	+	rod	+	nd
cps01.07 Ø	cream dome	+	rod	+	nd
actino7	***Actinomyces hordeovulneris***	**99.0**	***Actinomyces hordeovulneris***	**99.0**	**440**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.12 Ø	yellow convex	-	rod	+	nd
cps01.05* Ø	yellow dome	+	cocci chains	+	nd
cps01.22	white umbonate	-	rod	+	-
cps01.40	pink convex	nd	nd	nd	nd
actino8	**New species**	**NA**	***Actinomyces hordeovulneris***	**96.1**	**684**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.03*	small grey convex	+	rod short, clumps	+	-

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
cp10.24 Ø	cream pulvinate, red head	+	rod with granules	+	nd
actino9	***Actinomyces coleocanis***	**96.6**	***Actinomyces* sp.**	**96.6**	**605**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp17.02*	white shiny convex	-	rod short	-	-
arth1	***Rothia nasimurium***	**92.7**	***Rothia* sp.**	**92.7**	**361**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp09.03*	cream irregular pulvinate	+	cocci pairs	+	-
arth2	***Curtobacterium citreum* - like**	**95.2**	**Uncultured *Micrococcineae* bacterium**	**95.2**	**358**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.08	white convex	-	cocco-bacilli	-	-
cp03.01	grey convex	-	rod short	-	-
cp09.07*	small white convex	+	rod	-	-
arth3	***Micrococcus luteus***	**98.7**	***Micrococcus* sp.**	**98.8**	**814**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp06.27*	grey convex	+	cocci clumps	+	-
berg1	***Bergeyella zoohelcum***	**94.8**	***Bergeyella* sp.**	**97.3**	**868**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.10*	red convex	-	rod	+	+
cp06.10	red convex	-	rods + cocci	+	+
cp07.06	very small colourless convex	-	rod short	+	+
cp08.06	red/pink convex	-	cocco-bacilli pairs	+	-
cp08.18 Ø	light brown convex	-	rod variable	-	nd
cp08.21 Ø	pink convex	-	diplococci	+	nd
cp09.09	pink convex	-	rod fat	+	+
cps01.32	red convex	-	diplococci	+	+
cps01.39	brown/orange convex	-	rod long	+	+
berg2	***Bergeyella zoohelcum***	**95.5**	***Bergeyella* sp.**	**98.8**	**611**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.08	white convex	-	cocco-bacilli	+	+
cp08.07*	pink convex	-	rod short	+	-
bpp1	***Porphyromonas Catoniae***	**90.1**	***Porphyromonas* sp. oral clone**	**90.6**	**402**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.16* Ø	light yellow convex	-	cocco-bacilli	-	nd
cp03.12 Ø	grey convex large	nd	nd	+	nd
cp07.15 Ø	yellow pulvinate	-	cocci	+	nd
cp07.19 Ø	pink pulvinate	-	rod short	+	nd
cp09.17 Ø	yellow convex	-	cocco-bacilli	+	nd
bpp10	***Porphyromonas endodontalis***	**85.1**	***Porphyromonas* sp. oral clone**	**85.4**	**336**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp62.16* Ø	small black convex	-	cocci	-	nd
bpp11	***Porphyromonas macacae***	**95.4**	***Porphyromonas macacae***	**95.4**	**334**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.21 Ø	red pulvinate	-	rod short	-	nd
cp02.23* Ø	pink convex	-	rod short	-	nd

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
cp62.08 Ø	white/grey shiny convex	-	diplococci	+	nd
cp62.12 Ø	yellow/cream shiny convex	-	diplococci	+	nd
bpp12	***Stenotrophomonas* - like**	**91.8**	**Ultramicrobacterium str. DY01**	**94.1**	**913**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.07* Ø	light brown convex	-	rod long	+	nd
bpp2	***Prevotella* sp.**	**82.5**	***Prevotella* sp. oral clone**	**95.0**	**413**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.13* Ø	grey convex	-	rod large	-	nd
bpp3	***Prevotella heparinolytica***	**96.9**	***Prevotella heparinolytica***	**96.9**	**399**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.14 Ø	grey convex	-	cocci clumps	-	nd
cp02.19 Ø	yellow star shape	-	rod long	+	nd
cp05.15* Ø	white irregular	nd	nd	-	nd
cp05.17 Ø	yellow convex	-	rod	-	nd
bpp4	**New species**	**NA**	***Bacteroides* sp.**	**98.6**	**833**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.11* Ø	yellow convex	-	rod long	-	nd
cp03.16 Ø	yellow pulvinate	-	cocci	-	nd
cp10.11 Ø	yellow shiny convex, radial lines	nd	nd	weak	nd
cp17.09 Ø	large cream shiny convex	-	rod large, variable shape	-	nd
cp62.11 Ø	white/cream shiny convex	-	rods, irregular shapes, clumped	-	nd
bpp5	**New species**	**NA**	***Bacteroides* sp.**	**92.4**	**702**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.20	colourless tiny	-	rod variable	-	-
cp05.22	small colourless	-	rod	-	-
cp05.24 Ø	grey convex	-	cocci	-	nd
cp05.25* Ø	colourless convex	-	rod	-	nd
cp10.26	white/grey convex	-	rod	-	-
bpp6	***Porphyromonas gulae***	**99.9**	***Porphyromonas gulae***	**99.9**	**1083**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.14 Ø	brown pulvinate	+	cocci small	+	nd
cp05.30* Ø	brown pulvinate	-	cocci	+	nd
cp06.28 Ø	yellow pulvinate	-	cocci	+	nd
cp17.08 Ø	pale brown pulvinate	-	cocco-bacilli pairs	+	nd
cp17.12 Ø	small light brown convex	-	cocci	+	nd
bpp7	***Porphyromonas canoris***	**96.7**	***Porphyromonas canoris***	**96.7**	**538**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.12 Ø	red pulvinate	-	rod	+	nd
cp05.13* Ø	light brown convex	-	rod	+	nd
cp10.18 Ø	red pulvinate	-	rod long. Lots of extracellular material	+	nd
bpp8	***Porphyromonas cangingivalis***	**99.1**	***Porphyromonas cangingivalis***	**99.1**	**655**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.17 Ø	white pulvinate	-	rod	+	nd
cp01.25 Ø	orange irregular	nd	nd	+	nd
cp01.26 Ø	orange pulvinate	nd	nd	+	nd
cp01.27 Ø	red pulvinate	nd	nd	+	nd

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
cp02.16 Ø	yellow pulvinate	-	cocci clumps	+	nd
cp02.25 Ø	pink umbilicate	nd	nd	+	nd
cp02.26 Ø	orange pulvinate	nd	nd	+	nd
cp02.27 Ø	red pulvinate	-	cocci	+	nd
cp03.19* Ø	red pulvinate	nd	nd	+	nd
bpp9	***Porphyromonas cansulci***	**98.5**	***Porphyromonas cansulci***	**98.5**	**611**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.24 Ø	black convex	-	cocci	-	nd
cp62.15* Ø	dark brown pulvinate	-	diplococci	-	nd
bul1	**New genus**	**NA**	***Solobacterium* sp. oral clone**	**84.6**	**655**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp07.24* Ø	small grey convex	-	rod chains	-	nd
camp1	**New species**	**95.1**	***Campylobacter rectus***	**95.1**	**625**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.16 Ø	colourless convex	-	rod	+	nd
cp06.17 Ø	colourless convex	nd	nd	+	nd
cp06.20* Ø	clear/yellow convex	nd	nd	+	nd
cp07.14 Ø	red convex	nd	nd	+	nd
cp08.16 Ø	brown convex	-	rod small	+	nd
cp09.18 Ø	brown convex	-	rod short	+	nd
cp10.17 Ø	brown convex	-	rod short	+	nd
cp62.10 Ø	pale orange shiny convex	-	rods, small	+	nd
camp2	***Campylobacter curvus***	**96.7**	***Campylobacter curvus***	**96.7**	**374**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.26* Ø	brown convex	nd	nd	-	nd
capno1	***Capnocytophaga cynodegmi***	**97.4**	***Capnocytophaga cynodegmi***	**97.7**	**270**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cps01.44* Ø	small colourless convex	nd	nd	nd	nd
capno2	**New species**	**NA**	***Capnocytophaga* sp. oral strain**	**89.3**	**1065**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.19*	colourless convex	-	rod	-	-
cp09.10	red/brown convex	-	rod long	-	-
cp09.22 Ø	small colourless convex	-	rod long	-	nd
card1	**New sp.***	**NA**	***Cardiobacterium valvarum***	**93.0**	**890**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp06.01	grey convex	-	rod short, pairs	weak	+
cp06.05	brown convex, mycelial border	-	rods short, pairs	+	+
cp06.29 Ø	grey convex	-	cocco-bacilli	nd	nd
cp07.20* Ø	colourless convex	nd	nd	nd	nd
cp08.24	brown convex	-	rod short, pairs	+	-
clos1	***Clostridium celerecrescens***	**94.4**	***Firmicutes* sp. oral clone**	**97.1**	**421**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp03.21* Ø	colourless convex	nd	nd	-	nd
clos2	***Clostridium perfringens***	**100.0**	***Clostridium perfringens***	**100.0**	**304**

Table A.1 – Continued from previous page

Phylotype	**Consensus ID**	**%**	**Closest BLAST**	**%**	**bp**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cps01.17* Ø	large Irregular spreading yellow disc	-	cocci clumps	-	nd
clos3	***Clostridium* sp.**	**85.5**	***Peptococcus* sp. oral clone**	**95.2**	**470**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.19* Ø	colourless convex	-	rod	-	nd
cp01.22 Ø	grey filamentous convex	-	rod	-	nd
clos4	***Clostridium* sp.**	**93.1**	**Uncultured clone p-83-a5**	**94.2**	**175**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp17.14* Ø	small colourless convex	-	rod	-	nd
cory1	**New genus**	**NA**	***Corynebacterium bovis***	**76.3**	**683**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp03.20 Ø	white convex	nd	nd	+	nd
cp04.13	brown convex	-	cocco-bacilli pairs	+	-
cp04.17* Ø	small yellow convex	+	cocci	+	nd
cp10.28	brown convex	-	rod short	-	-
cory2	***Corynebacterium* sp.**	**95.4**	***Corynebacterium falsenii***	**95.4**	**838**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.04	orange convex	-	rod	+	-
cp04.08* Ø	cream convex	+	cocci	+	nd
cp06.03	cream convex	+	rod short	+	-
cp07.03	yellow shiny convex	+	rod short, pairs	+	-
cps01.30	white irregular raised curved	-	rod	+	-
cps01.34	white umbonate haemolytic	-	rod	+	-
cory3	**New species**	**NA**	***Corynebacterium ciconiae***	**93.1**	**851**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.10	white irregular pulvinate	nd	nd	+	-
cp03.02	white irregular convex curled	+	rod	+	-
cp03.18 Ø	white irregular	-	rod	+	nd
cp04.02*	cream convex	+	rod short	+	-
cp04.11	cream convex	-	rod short	+	-
cp04.14 Ø	cream convex	+	rod	+	nd
cp04.16 Ø	white convex	+	rod	+	nd
cp06.09	white irregular pulvinate	+	rod	+	-
cp07.04	yellow convex textured	+	rod	+	-
cp10.05	cream convex	+	rod	+	-
cory4	**New species**	**NA**	***Corynebacterium macginleyi***	**94.1**	**737**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.05*	cream/pink pulvinate	+	cocco bacilli pairs	+	-
cory5	**Corynebacterium appendicis***	**98.0**	**Corynebacterium appendicis**	**98.0**	**885**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp08.25*	small yellow convex	+	cocci	+	-
cory6	***Corynebacterium* sp.**	**94.2**	***Corynebacterium sundsvallense***	**94.2**	**135**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp10.09*	yellow pulvinate	+	rod long	+	-

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
curt1	**New species**	**NA**	***Curtobacterium* sp.**	**94.0**	**639**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp03.03*	white convex	-	rod	-	-
dia1	***Dialister invisus***	**99.8**	***Dialister invisus***	**99.8**	**758**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp17.10* Ø	grey raised	-	cocci small	-	nd
fuso1	***Filifactor alocis***	**99.0**	***Fusobacterium alocis***	**99.0**	**808**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp62.14* Ø	colourless raised, irregular edge	-	rods, short	-	nd
fuso2	**New genus**	**NA**	**Uncultured clone p-379-o3**	**85.9**	**420**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp03.14* Ø	grey convex filamentous	-	rod variable	-	nd
fuso3	**New species**	**NA**	***Fusobacterium nucleatum***	**96.1**	**1101**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp06.22* Ø	yellow irregular pulvinate	nd	nd	-	nd
cp62.07 Ø	yellow irregular convex shiny	-	rods, long, poorly stained	-	nd
fuso4	***Filifactor villosus***	**98.1**	***Filifactor villosus***	**98.1**	**384**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.20 Ø	yellow irregular curled	-	rod long	+	nd
cp03.17 Ø	yellow irregular	-	rod	+	nd
cp05.28* Ø	brown filamentous	-	rod	+	nd
cp06.19 Ø	yellow convex fuzzy	nd	nd	+	nd
gem1	***Gemella palaticanis***	**97.9**	***Gemella palaticanis***	**97.9**	**908**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.09 Ø	colourless raised	-	cocci pairs/tetrads	-	nd
cp04.15 Ø	colourless flat irregular	-	kidney shape pairs	-	nd
cp04.19* Ø	colourless flat irregular	-	kidney shape pairs	-	nd
haem1	**New species**	**NA**	***Haemophilus haemoglobinophilus***	**93.3**	**843**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp03.08	colourless umbonate	-	rod long, chains	+	-
cp05.08*	white/clear irregular	-	rod short	+	-
cp06.13	yellow/brown raised disc, irregular surface	-	cocci chains	+	+
cp06.21 Ø	colourless raised	nd	nd	+	nd
haem2	***Haemophilus haemoglobinophilus***	**96.60**	***Haemophilus haemoglobinophilus***	**96.60**	**275**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp62.03*	shiny grey raised	-	rods, very short (cocci-like) to medium length	nd	nd
haem3	***Haemophilus* sp.**	**95.5**	***Haemophilus* sp. oral clone**	**95.5**	**410**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.05	colourless raised	-	rod	+	-
cp03.04*	colourless convex	+	cocci	-	-
cp62.06	matt grey sunken convex	-	diplococci small	-	-

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
lac1	**New species**	**NA**	***Streptococcus infantarius* subsp. Infantarius**	**90.4**	**580**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cps01.14* Ø	colourless irregular disc	+	diplococci chains	-	nd
lam1	***Lampropedia hyalina***	**97.0**	***Lampropedia hyalina***	**97.0**	**336**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.01*	cream pulvinate	-	rod large	+	-
lep1	**New species**	**NA**	***Leptotrichia* sp. oral clone**	**93.9**	**687**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.18* Ø	brown pulvinate	-	rod long	-	nd
cp06.24 Ø	brown pulvinate	nd	nd	-	nd
cp07.16 Ø	brown pulvinate	nd	nd	+	nd
cp07.22	brown/green umbonate	-	rod long	-	nd
cp09.19 Ø	green pulvinate	-	rod long	-	nd
morax1	***Moraxella sp.***	**89.4**	**Uncultured clone 33-PA**	**90.1**	**728**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp05.23*	brown flat irregular	-	rod large	+	+
cp10.08	colourless convex	nd	rod large (unstained)	+	+
cp10.13 Ø	grey shallow convex	-	rod v. Large	+	nd
morax2	***Moraxella* sp.**	**95.1**	***Moraxella cuniculi***	**95.1**	**654**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp09.06	white/cream convex	-	rod - large irregular	weak	+
cp09.14* Ø	white convex	-	rod large	weak	nd
cp10.22 Ø	small colourless convex	nd	nd	+	nd
nd	**no sequence available**				
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.04	colourless raised	-	cocci tetrad clumps	-	-
cp01.05	white pulvinate curled	nd	nd	+	-
cp01.06	yellow raised	-	diplococci	+	-
cp01.28 Ø	white convex	nd	nd	-	nd
cp02.09	colourless convex	+	cocci	+	-
cp02.20 Ø	red pulvinate	-	cocco-bacilli	-	nd
cp02.28 Ø	colourless convex	nd	nd	nd	nd
cp03.10	pink convex	-	diplococci	+	+
cp04.03	yellow/brown concentric ridges	-	cocci	+	+
cp05.09	orange pulvinate	nd	nd	nd	nd
cp05.10	cream flat, yellow centre	-	rod short	-	-
cp05.11	red convex	-	rod	+	-
cp05.29 Ø	brown convex	-	rod	-	nd
cp06.04	brown convex	-	cocco bacilli pairs	+	-
cp06.06	cream curled	-	cocci	+	-
cp06.08	cream raised	-	cocci	+	-
cp06.30 Ø	light brown flat irregular	+	rod long	nd	nd
cp06.32 Ø	dark red pulvinate	nd	nd	nd	nd
cp07.11 Ø	yellow translucent convex	nd	nd	+	nd
cp08.05	yellow convex	+	rod short, pairs	+	-
cp09.11	brown/colourless convex	nd	nd	+	+
cp10.21 Ø	white/grey convex textured	+	rod, irregular clusters	+	nd
cp10.27	grey raised	nd	nd	+	-
cp62.13 Ø	light brown shiny pulvinate	-	diplococci	+	nd
cps01.01 Ø	yellow disc	+	cocco-bacilli	+	nd
cps01.25	small white convex	-	cocci	+	-
cps01.27	orange umbonate	-	cocci	+	-

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
cps01.29	irregular colourless flat	nd	nd	nd	nd
cps01.41	umbonate, yellow centre, white surround	nd	nd	nd	nd
cps01.43	yellow/black irregular raised	nd	nd	nd	nd
nei1	**New species**	**NA**	***Neisseria dentiae***	**96.0**	**619**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.07	yellow convex	-	cocci/rods	+	+
cp01.11	white irregular curled	-	cocco-bacilli pairs	+	+
cp03.06	orange convex	-	diplococci	+	+
cp04.01	yellow/brown irregular	-	cocci	+	+
cp05.02	brown conventric curled	-	cocci	+	+
cp06.15	brown convex	nd	nd	+	nd
cp07.10	yellow/brown concentric raised	-	cocci	+	+
cp07.21	colourless raised	-	rod curly	+	nd
cp08.04	orange convex	-	cocci clumps	+	-
cp08.09	cream concentric	-	cocci	+	+
cp08.10	yellow irregular pulvinate	-	cocci	+	+
cp09.02	yellow/brown concentric curled	-	cocci / kidney pairs	+	+
cp09.13	yellow convex	-	cocci, mostly pairs	+	nd
cp17.04*	large shiny orange convex, brown edge	-	cocco-bacilli pairs	+	+
cps01.20	yellow flat	-	cocci	+	+
nei3	***Neisseria canis***	**99.5**	***Neisseria canis***	**99.5**	**663**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.03	yellow convex	+	diplococci	+	+
cp02.07	orange convex	-	diplococci	+	+
cp03.09	grey convex	-	rod	+	-
cp03.11	yellow convex	-	diplococci	+	-
cp09.01	yellow/brown irregular curled	-	cocci	+	+
cp09.08	orange convex	-	cocco-bacilli pairs + tetrads	+	+
62.01*	yellow brown concentric ripple	nd	nd	nd	nd
cp62.02	orange shiny convex	-	diplococci, small	+	-
nei4	***Neisseria canis***	**98.6**	***Neisseria canis***	**98.6**	**1089**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.09	orange convex	-	rod	+	-
cp05.07*	orange convex	-	cocci pairs	+	-
cp06.31	colourless convex	nd	nd	+	nd
cp07.07	orange shiny convex	-	cocci	+	+
cp08.11	orange/brown convex	nd	nd	+	-
cp08.12	brown convex	nd	nd	+	-
cps01.16	Lg. White frilly disc	+	cocci clumps	weak	nd
nei5	***Neisseria weaveri***	**100.0**	***Neisseria weaveri***	**100.0**	**347**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.10	orange convex	-	diplococci	+	-
cp06.07	orange matt convex	+	cocci clumps	+	-
cp08.01	large brown/orange raised	-	rod	+	+
cp08.26	colourless flat irregular	-	rod	+	+
cp09.16*	orange convex	-	rod	+	nd
cp10.02	orange concentric raised	-	rod short	+	+
cp17.05	large grey raised shiny, pimpled orange centre	-	rod, variable length, clumps	+	+
cp62.05	yellow irregular	-	cocci	+	nd
cps01.28	orange rippled	-	cocci	+	+
cps01.33	large brown flat	-	rod variable	+	+

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
nei7	**New species**	**NA**	***Neisseria elongata* subsp. Glycolytica**	**94.4**	**702**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp07.09*	colourless irregular raised	-	rod short	+	+
cp08.20	colourless flat irregular	-	rod short	-	nd
nei8	***Xanthomonas* sp.**	**95.0**	***Xanthomonas* sp.**	**95.0**	**876**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.18 Ø	colourless convex	-	diplococci	+	nd
cp06.11*	yellow pulvinate	-	diplococci	+	+
past1	**New species**	**NA**	***Pasteurella dagmatis***	**98.5**	**718**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.22 Ø	grey flat	-	rod long	+	nd
cp06.12	colourless irregular	-	rod short	-	-
cp06.26	colourless filamentous	-	cocci	-	-
cp07.08*	yellow/brown raised disc	-	cocci	+	+
cp08.02	grey/green raised	-	cocci	+	-
cp10.04	cream raised/convex	-	rod short	+	-
past2	***Pasteurella dagmatis***	**98.3**	***Pasteurella dagmatis***	**98.3**	**820**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp03.07	yellow raised	-	cocci	+	-
cp03.13 Ø	grey convex	-	rod long	+	nd
cp07.12* Ø	larger yellow convex	nd	nd	+	nd
cp08.14 Ø	yellow raised translucent	nd	nd	+	nd
cp09.04	grey/green raised disc	-	rod	+	-
cp09.05	white/cream raised disc	-	cocco-bacilli	+	-
cp09.12 Ø	yellow convex, translucent edge	-	rod variable	+	nd
past3	**Bisgaard taxon 16**	**97.7**	**Bisgaard Taxon 16**	**97.7**	**839**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.01	grey raised β haemolytic	-	diplococci	+	-
cp01.12 Ø	grey umbonate	-	rod	+	nd
cp02.01*	grey raised β haemolytic	-	cocci	+	-
cp02.06	yellow raised β haemolytic	+	diplococci	+	-
cp03.05	grey raised β haemolytic	-	diplococci	+	-
cp08.03	shiny brown raised	-	cocci large	+	-
cp10.03	grey / green raised	-	rod	+	-
cp10.12 Ø	grey raised	-	rod, lots of extracellular material	+	nd
pep1	***Peptostreptococcus anaerobius***	**98.7**	***Peptostreptococcus anaerobius***	**98.7**	**333**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp10.14 Ø	shiny cream convex	+	cocci	-	-
cp10.20* Ø	cream pulvinate	+	cocci	-	nd
cp17.11 Ø	yellow pulvinate	+	cocci pairs in chains	-	nd
cp62.09 Ø	large yellow convex	+	rods, short	-	nd
pep2	***Micromonas micros***	**96.0**	***Peptostreptococcus* sp.**	**96.0**	**703**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp08.17 Ø	white pulvinate	-	cocci small	-	nd
cp09.15* Ø	small white convex	+	cocci small	-	nd
cp10.29 Ø	grey convex	-	cocci	-	nd
pep3	**New species**	**NA**	***Helcococcus* sp.**	**89.5**	**696**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
cp10.23* Ø	small white convex	-	cocci clumps	-	nd
prop1	***Tessaracoccus* sp.**	**93.5**	***Tessaracoccus bendigo-niensis***	**93.5**	**592**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp10.25*	brown convex	+	rod irregular	+	-
prop2	**New species**	**NA**	**Uncultured soil bacterium clone HN1-55**	**92.7**	**654**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.24 Ø	pink pulvinate	nd	nd	+	nd
cp07.25* Ø	grey/brown convex	-	cocci	+	nd
cps01.23	white irregular raised	-	rod	+	-
prop3	***Propionibacterium acnes***	**99.8**	***Propionibacterium acnes***	**99.8**	**696**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp09.20* Ø	pink pulvinate + rod short + nd				
pvib1	**New species**	**NA**	***Propionivibrio dicarboxylicus***	**93.2**	**607**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.18* Ø	colourless flat filamentous	-	rod	-	nd
rhod1	***Dietzia daqingensis***	**98.7**	***Dietzia daqingensis***	**98.7**	**417**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp03.22*	orange convex	+	diplococci clumps	nd	nd
staph1	***Staphylococcus epidermidis***	**99.6**	***Staphylococcus epidermidis***	**99.6**	**638**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cps01.35*	large white flat haemolytic	+	cocci clumps	+	-
cps01.36	large white flat	+	cocci clumps	+	-
strep1	***Streptococcus* sp.**	**99.4**	**Uncultured *Streptococcus* clone**	**99.4**	**343**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cps01.02 Ø	white disc	+	cocci	-	nd
cps01.42*	light brown irregular pulvinate curled	-	rod long	nd	nd
strep2	***Streptococcus minor***	**98.3**	***Streptococcus minor***	**98.3**	**634**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp01.23 Ø	white irregular curled	+	cocci clumps	nd	nd
cp02.21* Ø	white/yellow flat irregular	-	cocci	-	nd
cp03.15 Ø	white convex	-	cocci	+	nd
cps01.11 Ø	grey disc	-	cocco-bacilli	+	nd
strep3	***Streptococcus bovis***	**99.2**	***Streptococcus bovis***	**99.2**	**404**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cps01.15* Ø	cream frilly disc	+	rod	-	nd
strep4	**New species**	**NA**	***Streptococcus bovis***	**91.8**	**703**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.06*	orange/brown raised irregular	+	short rods, chains joined at long edge	-	-

Table A.1 – Continued from previous page

Phylotype	Consensus ID	%	Closest BLAST	%	bp
cps01.13 Ø	yellow star	+	cocci	weak	nd
cps01.26	small colourless convex	-	cocci clumps	+	-
cps01.31	colourless flat	-	cocci chains	-	-
cps01.37	irregular grey flat curled	-	cocci chains	-	-
wol1	***Wolinella succinogenes***	**98.5**	***Wolinella succinogenes***	**98.5**	**857**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp02.17* Ø	colourless convex	nd	nd	-	nd
xen1	***Xenophilus* sp.**	**94.8**	**Gram negative bacterium B3P-1**	**94.1**	**572**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp08.19* Ø	colurless raised	+	very small cocci	+	nd
xen2	**New species**	**92.9**	***Comamonadaceae* bacterium**	**94.3**	**855**
Isolates	Colony morphology	Gram-stain / morphology		Cat	Ox
cp04.12*	clear/brown flat	-	cocci	+	nd

Table A.1: Summary of phenotypic data for all isolates, grouped by phylotype. For each phylotype the isolate sequenced to the highest standard is indicated by an asterisk, and the closest match on GenBank to this isolate is indicated by the Closest BLAST entry for the phylotype, along with a percentage match and the number of bases sequenced. For each phyltype the consensus ID is also indicated, along with a percent match to the consensus ID based on a BLAST search of GenBank. The consensus ID takes into account all available data for all isolates belonging to the phylotype, and is considered the most reliable identification for the phylotype as a whole. Phenotypic data includes catalase (Cat) and oxidase (Ox) reactions, colony morphology, and Gram-stain reaction and morphology. Ø next to the isolate name indicates anaerobic isolation, otherwise isolates were obtained aerobically.

Bibliography

Allaker RP, Young KA, Langlois T, de Rosayro R, and Hardie JM. Dental plaque flora of the dog with reference to fastidious and anaerobic bacteria associated with bites. *J Vet Dent*, 14:127–130, 1997a.

Allaker RP, de Rosayro R, Young KA, and Hardie JM. Prevalence of *Porphyromonas* and *Prevotella* species in the dental plaque of dogs. *Vet Rec*, 140:147–148, 1997b.

Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ. Basic local alignment search tool. *J Mol Biol*, 215:403–410, 1990.

Amano H, Akimoto T, Takahashi KW, Nakagawa M, and Saito M. Isolation of *Corynebacterium kutscheri* from aged Syrian hamsters (*Mesocricetus auratus*). *Lab Anim Sci*, 41:265–268, 1991.

Amao H, Akimoto T, Komukai Y, Sawada T, Saito M, and Takahashi KW. Detection of *Corynebacterium kutscheri* from the oral cavity of rats. *Exp Anim*, 51:99–102, 2002.

Anderl JN, Franklin MJ, and Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother*, 44:1818–1824, 2000.

Baar C, Eppinger M, Raddatz G, Simon J, Lanz C, Klimmek O, Nandakumar R, Gross R, Rosinus A, Keller H, Jagtap P, Linke B, Meyer F, Lederer H, and Schuster SC. Complete genome sequence and analysis of *Wolinella succinogenes*. *Proc Natl Acad Sci U S A*, 100:11690–11695, 2003.

Bakken LR. Separation and purification of bacteria from soil. *Appl Environ Microbiol.*, 49(6):1482–1487, 1985.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches SK, Dewhirst FE, and Griffen AL. Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol*, 40:1001–1009, 2002.

Beighton D and Miller WA. A microbiological study of normal flora of macropod dental plaque. *J Dent Res*, 56:995–1000, 1977.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, and Wheeler DL. GenBank: update. *Nucleic Acids Res*, 32(Database issue):D23–D26, 2004.

Bradshaw DJ, Homer KA, Marsh PD, and Beighton D. Metabolic cooperation in oral microbial communities during growth on mucin. *Microbiology*, 140:3407–3412, 1994.

Bradshaw DJ, Marsh PD, Watson GK, and Allison C. Role of *Fusobacterium nucleatum* and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration. *Infect Immun*, 66:4729–4732, 1998.

Brinig MM, Lepp PW, Ouverney CC, Armitage GC, and Relman DA. Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. *Appl Environ Microbiol*, 69:1687–1694, 2003.

Busscher HJ and van der Mei HC. Use of flow chamber devices and image analysis methods to study microbial adhesion. *Methods Enzymol*, 253:455–477, 1995.

Busscher HJ, Doornbusch GI, and der Mei HCV. Adhesion of mutans streptococci to glass with and without a salivary coating as studied in a parallel-plate flow chamber. *J Dent Res*, 71:491–500, 1992.

Butzler JP. *Campylobacter*, from obscurity to celebrity. *Clin Microbiol Infect*, 10:868–876, 2004.

Carandina G, Bacchelli M, Virgili A, and Strumia R. *Simonsiella* filaments isolated from erosive lesions of the human oral cavity. *J Clin Microbiol*, 19:931–933, 1984.

Cato EP, Moore LVH, and Moore WEC. *Fusobacterium alocis* sp. nov. and *Fusobacterium sulci* sp. nov. from the human gingival sulcus. *Int J Syst Bacteriol*, 35:475–477, 1985.

Chan EC and McLaughlin R. Taxonomy and virulence of oral spirochetes. *Oral Microbiol Immunol*, 15:1–9, 2000.

Chávez de Paz LE, Molander A, and Dahlén G. Gram-positive rods prevailing in teeth with apical periodontitis undergoing root canal treatment. *Int Endod J*, 37:579–587, 2004.

Chen CK and Wilson ME. *Eikenella corrodens* in human oral and non-oral infections: a review. *J Periodontol*, 63:941–953, 1992.

Chen C. Distribution of a newly described species, *Kingella oralis*, in the human oral cavity. *Oral Microbiol Immunol*, 11:425–427, 1996.

Christensen H, Bisgaard M, Frederiksen W, Mutters R, Kuhnert P, and Olsen JE. Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify recommendation 30b of the Bacteriological Code (1990 Revision). *Int J Syst Evol Microbiol*, 51:2221–2225, 2001.

Ciantar M, Spratt DA, Newman HN, and Wilson M. *Capnocytophaga granulosa* and *Capnocytophaga haemolytica*: novel species in subgingival plaque. *J Clin Periodontol*, 28:701–705, 2001.

Cisar JO, Kolenbrander PE, and McIntire FC. Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. *Infect Immun*, 24:742–752, 1979.

Clark JW, Hossain S, Burnside CA, and Kambhampati S. Coevolution between a cockroach and its bacterial endosymbiont: a biogeographical perspective. *Proc R Soc Lond B Biol Sci*, 268:393–398, 2001.

Colby SM and Russell RRB. Sugar metabolism by mutans streptococci. *Soc Appl Bacteriol Symp Ser*, 83:80S–88S, 1997.

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, and Tiedje JM. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res*, 31:442–443, 2003.

Collins MD and Lawson PA. The genus *Abiotrophia* (Kawamura et al.) is not monophyletic: proposal of *Granulicatella* gen. nov., *Granulicatella adiacens* comb. nov., *Granulicatella elegans* comb. nov. and *Granulicatella balaenopterae* comb. nov. *Int J Syst Evol Microbiol*, 50:365–369, 2000.

Collins MD, Jovita MR, Foster G, Sjdn B, and Falsen E. Characterization of a *Gemella*-like organism from the oral cavity of a dog: description of *Gemella palaticanis* sp. nov. *Int J Syst Bacteriol*, 49:1523–1526, 1999.

Collins MD, Hoyles L, Hutson RA, Foster G, and Falsen E. *Corynebacterium testudinoris* sp. nov., from a tortoise, and *Corynebacterium felinum* sp. nov., from a Scottish wild cat. *Int J Syst Evol Microbiol*, 51:1349–1352, 2001.

Conrads G, Claros MC, Citron DM, Tyrrell KL, Merriam V, and Goldstein EJ. 16S-23S rDNA internal transcribed spacer sequences for analysis of the phylogenetic relationships among species of the genus *Fusobacterium*. *Int J Syst Evol Microbiol*, 52:493–499, 2002.

Contreras A, Doan N, Chen C, Rusitanonta T, Flynn MJ, and Slots J. Importance of *Dialister pneumosintes* in human periodontitis. *Oral Microbiol Immunol*, 15:269–272, 2000.

Crociani F, Biavati B, Alessandrini A, Chiarini C, and Scardovi V. *Bifidobacterium inopinatum* sp. nov. and *Bifidobacterium denticolens* sp. nov., two new species isolated from human dental caries. *Int J Syst Bacteriol*, 46:564–571, 1996.

Cześnikiewicz-Guzik M, Karczewska E, Bielański W, Guzik TJ, Kapera P, Targosz A, Konturek SJ, and Loster B. Association of the presence the *Helicobacter pylori* in the oral cavity and in the stomach. *J Physiol Pharmacol*, pages 105–115, 2004.

Dahle UR, Tronstad L, and Olsen I. Spirochaetes in oral infections. *Endod Dent Traumatol*, 9:87–94, 1993.

Darout IA, Albandar JM, and Skaug N. Correlations between bacterial levels in autologous subgingival plaque and saliva of adult Sudanese. *Clin Oral Investig.*, 6:210–216, 2002.

Debelian GJ, Olsen I, and Tronstad L. Systemic diseases caused by oral microorganisms. *Endod Dent Traumatol*, 10:57–65, 1994.

DeLong EF, Wickham GS, and Pace NR. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science*, 243:1360–1363, 1989.

DeLong EF. *Archaea* in coastal marine environments. *Proc Natl Acad Sci U S A*, 89:5685–5689, 1992.

Dent VE and Marsh PD. Evidence for a basic plaque microbial community on the tooth surface in animals. *Arch Oral Biol*, 26:171–179, 1981.

Dewhirst FE, Tamer MA, Ericson RE, Lau CN, Levanos VA, Boches SK, Galvin JL, and Paster BJ. The diversity of periodontal spirochetes by 16S rRNA analysis. *Oral Microbiol Immunol*, 15:196–202, 2000.

Dewhirst FE, Paster BJ, Tzellas N, Coleman B, Downes J, Spratt DA, and Wade WG. Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family *Coriobacteriaceae*: description of *Olsenella* gen. nov., reclassification of *Lactobacillus uli* as *Olsenella uli* comb. nov. and description of *Olsenella profusa* sp. nov. *Int J Syst Evol Microbiol*, 51:1797–1804, 2001.

Downes J, Munson MA, Spratt DA, Kononen E, Tarkka E, Jousimies-Somer H, and Wade WG. Characterisation of *Eubacterium*-like strains isolated from oral infections. *J Med Microbiol*, 50:947–951, 2001.

Downes J, Munson M, and Wade WG. *Dialister invisus* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol*, 53:1937–1940, 2003.

Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, and Raoult D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol*, 38:3623–3630, 2000.

Dzink JL, Socransky SS, and Haffajee AD. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol*, 15:316–323, 1988.

Elliott D, Harrison E, Handley PS, Ford SK, Jaffray E, Mordan N, and McNab R. Prevalence of Csh-like fibrillar surface proteins among mitis group oral streptococci. *Oral Microbiol Immunol*, 18:114–120, 2003.

Elliott D, Pratten J, Edwards M, Crowther J, Petrie A, and Wilson M. Bacterial Biofilm Development on Hydroxyapatite-Coated Glass. *Curr Microbiol*, 2005.

Eribe ER, Paster BJ, Caugant DA, Dewhirst FE, Stromberg VK, Lacy GH, and Olsen I. Genetic diversity of *Leptotrichia* and description of *Leptotrichia goodfellowii* sp. nov., *Leptotrichia hofstadii* sp. nov., *Leptotrichia shahii* sp. nov. and *Leptotrichia wadei* sp. nov. *Int J Syst Evol Microbiol*, 54:583–592, 2004.

Estreicher A, Broggiato A, Duroux P, Andersen E, and Cimasoni G. Low molecular-weight proteins in human gingival crevicular fluid. *Arch Oral Biol*, 41:733–738, 2004.

Felsenstein J. PHYLIP (Phylogeny Inference Package), version 3.5c. Computer program available from http://evolution.genetics.washington.edu/phylip.html. Published by Department of Genetics, University of Washington, Seattle., 1993.

Fischer RG, Edwardsson S, and Klinge B. Oral microflora of the ferret at the gingival sulcus and mucosa membrane in relation to ligature-induced periodontitis. *Oral Microbiol Immunol*, 9:40–49, 1994.

Forney LJ, Zhou X, and Brown CJ. Molecular microbial ecology: land of the one-eyed king. *Curr Opin Microbiol*, 7:210–220, 2004.

Forsblom B, Sarkiala-Kessel E, Kanervo A, Visnen ML, Helander M, and Jousimies-Somer H. Characterisation of aerobic gram-negative bacteria from

subgingival sites of dogs–potential bite wound pathogens. *J Med Microbiol*, 51:207–220, 2002.

Fournier D, Mouton C, Lapierre P, Kato T, Okuda K, and Mnard C. *Porphyromonas gulae* sp. nov., an anaerobic, gram-negative coccobacillus from the gingival sulcus of various animal hosts. *Int J Syst Evol Microbiol*, 51:1179–1189, 2001.

Fredericks DN and Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin Microbiol Rev*, 9:18–33, 1996.

Fujise O, Chen W, Rich S, and Chen C. Clonal diversity and stability of subgingival *Eikenella corrodens*. *J Clin Microbiol*, 42:2036–2042, 2004.

Gad T. Periodontal disease in dogs. I. Clinical investigation. *J Periodont Res*, 3:268–272, 1968.

Gafan GP, Elliott D, Wilson M, and Spratt D. Profiling the oral microbiota associated with the prevalence of three periodontal pathogens. In McBain A, Allison D, Pratten J, Spratt D, Upton M, and Verran J, editors, *Biofilms: Persistence and Ubiquity. Contributions made at the Seventh Meeting of the Biofilm Club held at Gregynog Hall, Powys.*, pages 323–333. the Biofilm Club, School of Pharmacy and Pharmaceutical Sciences, The University of Manchester, Manchester, UK., 2005.

Garland JL and Mills AL. Classification and Characterization of Heterotrophic Microbial Communities on the Basis of Patterns of Community-Level Sole-Carbon-Source Utilization. *Appl Environ Microbiol*, 57(8):2351–2359, 1991.

Garrity GM, Winters M, Kuo AW, and Searles DB. *Taxonomic Outline of the Prokaryotes. Bergey's Manual of Systematic Bacteriology, 2nd Edn.* Springer-Verlag, NY., 2002.

Gebara EC, Pannuti C, Faria CM, Chehter L, Mayer MP, and Lima LA. Prevalence of *Helicobacter pylori* detected by polymerase chain reaction in the oral cavity of periodontitis patients. *Oral Microbiol Immunol*, 19:277–280, 2004.

Genco RJ. Current view of risk factors for periodontal diseases. *J Periodontol*, 67:1041–1049, 1996.

Gibbons RJ and Nygaard M. Interbacterial aggregation of plaque bacteria. *Arch Oral Biol*, 15:1397–1400, 1970.

Gilbert P, Allison DG, Evans DJ, Handley PS, and Brown MR. Growth rate control of adherent bacterial populations. *Appl Environ Microbiol*, 55:1308–1311, 1989.

Gmür R and Thurnheer T. Direct quantitative differentiation between *Prevotella intermedia* and *Prevotella nigrescens* in clinical specimens. *Microbiology*, 148:1379–1387, 2002.

Goebel BM and Stackebrandt E. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl Environ Microbiol*, 60:1614–1621, 1994.

Goldstein EJ. Bite wounds and infection. *Clin Infect Dis*, 14:633–638, 1992.

Good NE, Winget GD, Winter W, Connolly TN, Izawa S, and Singh RM. Hydrogen ion buffers for biological research. *Biochemistry*, 5:467–477, 1966.

Greiner O, Day PJ, Altwegg M, and Nadal D. Quantitative detection of *Moraxella catarrhalis* in nasopharyngeal secretions by real-time PCR. *J Clin Microbiol*, 41:1386–1390, 2003.

Hale FA. Dental caries in the dog. *J Vet Dent*, 15:79–83, 1998.

Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.*, 41:95–98, 1999.

Hamp SE, Olsson SE, Madsen KF, Viklands P, and Fornell J. A macroscopic and radiologic investigation of dental diseases in dogs. *Veterinary Radiology*, 25:86–92, 1984.

Hamp SE, Hamp M, Olsson SE, Lindberg R, and Schauman P. Radiography of spontaneous periodontitis in dogs. *J Periodont Res*, 32:589–597, 1997.

Han XY, Meltzer MC, Woods JT, and Fainstein V. Endocarditis with ruptured cerebral aneurysm caused by *Cardiobacterium valvarum* sp. nov. *J Clin Microbiol*, 42:1590–1595, 2004.

Handley PS, Carter PL, Wyatt JE, and Hesketh LM. Surface structures (peritrichous fibrils and tufts of fibrils) found on *Streptococcus sanguis* strains may be related to their ability to coaggregate with other oral genera. *Infect Immun*, 47:217–227, 1985.

Hardham J, Dreier K, Wong J, Sfintescu C, and Evans RT. Pigmented-anaerobic bacteria associated with canine periodontitis. *Vet Microbiol*, 106:119–128, 2005.

Hardham JM, King KW, Dreier K, Wong J, Strietzel C, Eversole RR, Sfintescu C, and Evans RT. Transfer of *Bacteroides splanchnicus* to *Odoribacter* gen. nov. as *Odoribacter splanchnicus* comb. nov., and description of *Odoribacter denticanis* sp. nov., isolated from the crevicular spaces of canine periodontal disease patients. *Int. J. Syst. Evol. Microbiol.*, In Press.

Hardie JM and Whiley RA. Plaque microbiology of crown caries. In Newman HN and Wilson M, editors, *Dental plaque revisited: oral biofilms in health and disease*, pages 283–294. BioLine, Cardiff, UK, 1999.

Harper-Owen R, Dymock D, Booth V, Weightman AJ, and Wade WG. Detection of unculturable bacteria in periodontal health and disease by PCR. *J Clin Microbiol*, 37:1469–1473, 1999.

Harris JK, Kelley ST, and Pace NR. New perspective on uncultured bacterial phylogenetic division OP11. *Appl Environ Microbiol*, 70:845–849, 2004.

Harvey CE, Thornsberry C, and Miller BR. Subgingival bacteria–comparison of culture results in dogs and cats with gingivitis. *J Vet Dent*, 12:147–150, 1995.

Hedlund BP and Staley JT. Phylogeny of the genus *Simonsiella* and other members of the *Neisseriaceae*. *Int J Syst Evol Microbiol*, 52:1377–1382, 2002.

Henderson B, Nair SP, Ward JM, and Wilson M. Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. *Annu Rev Microbiol*, 57:29–55, 2003.

Hennet PR and Harvey CE. Anaerobes in periodontal disease in the dog: a review. *J Vet Dent*, 8:18–21, 1991a.

Hennet PR and Harvey CE. Spirochetes in periodontal disease in the dog: a review. *J Vet Dent*, 8:16–17, 1991b.

Hennet PR and Harvey CE. Aerobes in periodontal disease in the dog: a review. *J Vet Dent*, 8:9–11, 1991c.

Hillam DG and Hull PS. The influence of experimental gingivitis on plaque formation. *J Clin Periodontol*, 4:56–61, 1977.

Hodgson AE, Nelson SM, Brown MR, and Gilbert P. A simple *in vitro* model for growth control of bacterial biofilms. *J Appl Bacteriol*, 79:87–93, 1995.

Holt SC. Anatomy and chemistry of spirochetes. *Microbiol Rev*, 42:114–160, 1978.

Hope CK and Wilson M. Effects of dynamic fluid activity from an electric toothbrush on in vitro oral biofilms. *J Clin Periodontol*, 30:624–629, 2003.

Hoyles L, Falsen E, Foster G, Pascual C, Greko C, and Collins MD. *Actinomyces canis* sp. nov., isolated from dogs. *Int J Syst Evol Microbiol*, pages 1547–1551, 2000.

Huang X. A contig assembly program based on sensitive detection of fragment overlaps. *Genomics*, 14:18–25, 1992.

Hugenholtz P, Tyson GW, Webb RI, Wagner AM, and Blackall LL. Investigation of candidate division TM7, a recently recognized major lineage of the domain *Bacteria* with no known pure-culture representatives. *Appl Environ Microbiol*, 67:411–419, 2001.

Isogai E, Isogai H, Miura H, Takano K, Aoi Y, Hayashi M, and Namioka S. Oral flora of mongrel and beagle dogs with periodontal disease. *Nippon Juigaku Zasshi*, 51:110–118, 1989a.

Isogai H, Isogai E, Okamoto H, Shirakawa H, Nakamura F, Matsumoto T, Watanabe T, Miura H, Aoi Y, and Kagota W. Epidemiological study on periodontal diseases

and some other dental disorders in dogs. *Nippon Juigaku Zasshi*, 51:1151–1162, 1989b.

Isogai H, Kosako Y, Benno Y, and Isogai E. Ecology of genus *Porphyromonas* in canine periodontal disease. *Zentralbl Veterinarmed B*, 46:467–473, 1999.

Jalava J and Eerola E. Phylogenetic analysis of *Fusobacterium alocis* and *Fusobacterium sulci* based on 16S rRNA gene sequences: proposal of *Filifactor alocis* (Cato, Moore and Moore) comb. nov. and *Eubacterium sulci* (Cato, Moore and Moore) comb. nov. *Int J Syst Bacteriol*, pages 1375–1379, 1999.

Jukes TH and Cantor CR. Evolution of protein molecules. In Munro M, editor, *Mammalian Protein Metabolism*, pages 21–132. Academic Press, New York, 1969.

Kaster AG and Brown LR. Extracellular dextranase activity produced by human oral strains of the genus *Bifidobacterium*. *Infect Immun*, 42:716–720, 1983.

Kawamura Y, Hou XG, Sultana F, Miura H, and Ezaki T. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. *Int J Syst Bacteriol*, 45:406–408, 1995a.

Kawamura Y, Hou XG, Sultana F, Liu S, Yamamoto H, and Ezaki T. Transfer of *Streptococcus adjacens* and *Streptococcus defectivus* to *Abiotrophia* gen. nov. as *Abiotrophia adiacens* comb. nov. and *Abiotrophia defectiva* comb. nov., respectively. *Int J Syst Bacteriol*, 45:798–803, 1995b.

Kawamura Y, Whiley RA, Shu SE, Ezaki T, and Hardie JM. Genetic approaches to the identification of the mitis group within the genus *Streptococcus*. *Microbiology*, 145:2605–2613, 1999.

Kazor CE, Mitchell PM, Lee AM, Stokes LN, Loesche WJ, Dewhirst FE, and Paster BJ. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *J Clin Microbiol*, 41:558–563, 2003.

Kimura M. Molecular evolutionary clock and the neutral theory. *J Mol Evol*, 26:24–33, 1987.

Kinniment SL, Wimpenny JW, Adams D, and Marsh PD. Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology*, 142:631–638, 1996.

Kiwan Y, Shuhaiber H, and Chungh T. *Cardiobacterium hominis* endocarditis. *J Cardiovasc Surg (Torino)*, 30:281–283, 2004.

Kolenbrander PE and London J. Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol*, 175:3247–3252, 1993.

Kolenbrander PE, Andersen RN, and Holdeman LV. Coaggregation of oral *Bacteroides* species with other bacteria: central role in coaggregation bridges and competitions. *Infect Immun*, 48:741–746, 1985.

Kolenbrander PE, Andersen RN, and Moore LVH. Intrageneric Coaggregation among Strains of Human Oral Bacteria: Potential Role in Primary Colonization of the Tooth Surface. *Appl Environ Microbiol*, 56:3890–3894, 1990.

Kolenbrander PE, Parrish KD, Andersen RN, and Greenberg EP. Intergeneric coaggregation of oral *Treponema* spp. *Infect Immun*, 63:4584–4588, 1995.

Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, and Palmer RJ. Communication among oral bacteria. *Microbiol Mol Biol Rev*, 66:486–505, 2002.

Kornman KS, Siegrist B, Soskolne WA, and Nuki K. The predominant cultivable subgingival flora of beagle dogs following ligature placement and metronidazole therapy. *J Periodontal Res*, 16:251–258, 1981.

Kuhn DA, Gregory DA, Buchanan GE, Nyby MD, and Daly KR. Isolation, characterization, and numerical taxonomy of *Simonsiella* strains from the oral cavities of cats, dogs, sheep, and humans. *Arch Microbiol*, 118:235–241, 1978.

Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, and Leys EJ. New bacterial species associated with chronic periodontitis. *J Dent Res*, 82:338–344, 2003.

Kurnatowska AJ and Kurnatowski P. Trichomonosis of the oral cavity complicated by mycosis. *Parassitologia*, 40:339–342, 1998.

Lai CH, Males B, Dougherty P, Berthold P, and Listgarten M. *Centipeda periodontii*, gen. nov., sp. nov. from human periodontal lesions. *Int. J. Syst. Bacteriol.*, 33:628–635, 1983.

Lai CH, Oshima K, Slots J, and Listgarten MA. *Wolinella recta* in adult gingivitis and periodontitis. *J Periodontal Res*, 27:8–14, 1992.

Lane DJ. 16s/23s rrna sequencing. In Stackebrandt E and Goodfellow M, editors, *Nucleic acid techniques in bacterial systematics.*, pages 115–175. John Wiley & Sons, New York, N.Y., 1969.

Lee PP, Mak WY, and Newsome P. The aetiology and treatment of oral halitosis: an update. *Hong Kong Med J*, 10:414–418, 2004.

Lepp PW, Brinig MM, Ouverney CC, Palm K, Armitage GC, and Relman DA. Methanogenic *Archaea* and human periodontal disease. *Proc Natl Acad Sci U S A*, 101:6176–6181, 2004.

Lewis TM. Resistance of dogs to dental caries: a two-year study. *J Dent Res*, 44:1254, 1965.

Li WH, Gouy M, Sharp PM, O'hUigin C, and Yang YW. Molecular phylogeny of Rodentia, Lagomorpha, Primates, Artiodactyla, and Carnivora and molecular clocks. *Proc Natl Acad Sci U S A*, 87:6703–6707, 1990.

Lindhe J and Nyman S. The effect of plaque control and surgical pocket elimination on the establishment and maintenance of periodontal health. *J Clin Periodontol*, 2:67–79, 1975.

Lockhart PB. The risk for endocarditis in dental practice. *Periodontol 2000*, 23:127–135, 2000.

Löe H. The Gingival Index, the Plaque Index and the Retention Index Systems. *J Periodontol*, 38:Suppl:610–Suppl:616, 1967.

Loesche WJ. The role of spirochetes in periodontal disease. *Adv Dent Res*, 2:275–283, 1988.

López-García P, Duperron S, Philippot P, Foriel J, Susini J, and Moreira D. Bacterial diversity in hydrothermal sediment and *epsilonproteobacterial* dominance in experimental microcolonizers at the Mid-Atlantic Ridge. *Environ Microbiol*, 5:961–976, 2003.

Madden TE and Caton JG. Animal models for periodontal disease. *Methods Enzymol*, 235:106–119, 1994.

Marsh PD, Hunter JR, Bowden GH, Hamilton IR, McKee AS, Hardie JM, and Ellwood DC. The influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. *J Gen Microbiol*, 129:755–770, 1983.

Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology*, 149:279–294, 2003.

Matsuki T, Watanabe K, Tanaka R, Fukuda M, and Oyaizu H. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. *Appl Environ Microbiol*, 65:4506–4512, 1999.

McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, and Gilbert P. Growth and molecular characterization of dental plaque microcosms. *J Appl Microbiol*, 94:655–664, 2003.

McBain AJ, Sissons C, Ledder RG, Sreenivasan PK, Vizio WD, and Gilbert P. Development and characterization of a simple perfused oral microcosm. *J Appl Microbiol*, 98:624–634, 2005.

McCarthy C. Chromas. Computer program available from http://www.technelysium.com.au/chromas.html. Published by Griffith University, Brisbane, Australia, 1997.

McCoy WF, Bryers JD, Robbins J, and Costerton JW. Observations of fouling biofilm formation. *Can J Microbiol*, 27:910–917, 1981.

Mikkelsen L, Theilade E, and Poulsen K. *Abiotrophia species* in early dental plaque. *Oral Microbiol Immunol*, 15:263–268, 2000.

Moore LV and Moore WE. *Oribaculum catoniae* gen. nov., sp. nov.; *Catonella morbi* gen. nov., sp. nov.; *Hallella seregens* gen. nov., sp. nov.; *Johnsonella ignava* gen. nov., sp. nov.; and *Dialister pneumosintes* gen. nov., comb. nov., nom. rev., Anaerobic gram-negative bacilli from the human gingival crevice. *Int J Syst Bacteriol*, 44:187–192, 1994.

Moore WE, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, and Ranney RR. Bacteriology of moderate (chronic) periodontitis in mature adult humans. *Infect Immun*, 42:510–515, 1983.

Moore LV, Moore WE, Cato EP, Smibert RM, Burmeister JA, Best AM, and Ranney RR. Bacteriology of human gingivitis. *J Dent Res*, 66:989–995, 1987.

Munson MA, Banerjee A, Watson TF, and Wade WG. Molecular analysis of the microflora associated with dental caries. *J Clin Microbiol*, 42:3023–3029, 2004.

Muyzer G, de Waal EC, and Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol*, 59:695–700, 1993.

Nakazawa F, Sato M, Poco SE, Hashimura T, Ikeda T, Kalfas S, Sundqvist G, and Hoshino E. Description of *Mogibacterium pumilum* gen. nov., sp. nov. and *Mogibacterium vescum* gen. nov., sp. nov., and reclassification of *Eubacterium timidum* (Holdeman et al. 1980) as *Mogibacterium timidum* gen. nov., comb. nov. *Int J Syst Evol Microbiol*, 50:679–688, 2000.

Nakazawa F, Poco SE, Sato M, Ikeda T, Kalfas S, Sundqvist G, and Hoshino E. Taxonomic characterization of *Mogibacterium diversum* sp. nov. and *Mogibacterium neglectum* sp. nov., isolated from human oral cavities. *Int J Syst Evol Microbiol*, 52:115–122, 2002.

Nociti FH, Toledo RCD, Machado MA, Stefani CM, Line SR, and Gonalves RB. Clinical and microbiological evaluation of ligature-induced peri-implantitis and periodontitis in dogs. *Clin Oral Implants Res*, 12:295–300, 2001.

Noorda WD, Purdell-Lewis DJ, van Montfort AM, and Weerkamp AH. Monobacterial and mixed bacterial plaques of *Streptococcus mutans* and *Veillonella alcalescens* in an artificial mouth: development, metabolism, and effect on human dental enamel. *Caries Res*, 22:342–347, 1988.

Nyby MD, Gregory DA, Kuhn DA, and Pangborn J. Incidence of *Simonsiella* in the oral cavity of dogs. *J Clin Microbiol*, 6:87–88, 1977.

Nyvad B and Kilian M. Microbiology of the early colonization of human enamel and root surfaces *in vivo*. *Scand J Dent Res*, 95:369–380, 1987.

Ogino A, Koshikawa H, Nakahara T, and Uchiyama H. Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. *J Appl Microbiol*, 91:625–635, 2001.

Olsen I, Johnson JL, Moore LV, and Moore WE. *Lactobacillus uli* sp. nov. and *Lactobacillus rimae* sp. nov. from the human gingival crevice and emended descriptions of *lactobacillus minutus* and *Streptococcus parvulus*. *Int J Syst Bacteriol*, 41:261–266, 1991.

Orland FJ, Blayney JR, Harrison RW, Reyniers JA, Trexler PC, Wagner M, Gordon HA, and Luckey TD. Use of the germfree animal technic in the study of experimental dental caries. *J Dent Res*, 33:147–174, 1954.

Otto K and Silhavy TJ. Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proc Natl Acad Sci U S A*, 99:2287–2292, 2002.

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, and Dewhirst FE. Bacterial diversity in human subgingival plaque. *J Bacteriol*, 183:3770–3783, 2001.

Paster BJ, Russell MK, Alpagot T, Lee AM, Boches SK, Galvin JL, and Dewhirst FE. Bacterial Diversity in Necrotizing Ulcerative Periodontitis in HIV-Positive Subjects. *Ann Periodontol*, 7:8–16, 2002.

Potts TV, Zambon JJ, and Genco. RJ. Reassignment of *Actinobacillus actinomycetemcomitans* to the genus *Haemophilus* as *Haemophilus actinomycetemcomitans* comb. nov. *Int. J. Syst. Bacteriol.*, 35:337–341, 1985.

Pratten J and Wilson M. Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose. *Antimicrob Agents Chemother*, 43:1595–1599, 1999.

Pratten J, Wills K, Barnett P, and Wilson M. *In vitro* studies of the effect of antiseptic-containing mouthwashes on the formation and viability of *Streptococcus sanguis* biofilms. *J Appl Microbiol*, 84:1149–1155, 1998.

Pratten J, Wilson M, and Spratt DA. Characterization of *in vitro* oral bacterial biofilms by traditional and molecular methods. *Oral Microbiol Immunol*, 18:45–49, 2003.

Puech V, Chami M, Lemassu A, Lanelle MA, Schiffler B, Gounon P, Bayan N, Benz R, and Daff M. Structure of the cell envelope of corynebacteria: importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane. *Microbiology*, 147:1365–1382, 2001.

Rams TE, Feik D, Young V, Hammond BF, and Slots J. Enterococci in human periodontitis. *Oral Microbiol Immunol*, 7:249–252, 1992.

Rasband W. ImageJ. Computer program available from http://rsb.info.nih.gov/ij/. Published by U. S. National Institutes of Health, Bethesda,Maryland, USA., 2005.

Ready D, Lancaster H, Qureshi F, Bedi R, Mullany P, and Wilson M. Effect of amoxicillin use on oral microbiota in young children. *Antimicrob Agents Chemother*, 48:2883–2887, 2004.

Rickard AH, Leach SA, Buswell CM, High NJ, and Handley PS. Coaggregation between aquatic bacteria is mediated by specific-growth-phase-dependent lectin-saccharide interactions. *Appl Environ Microbiol*, 66:431–434, 2000.

Rickard AH, McBain AJ, Ledder RG, Handley PS, and Gilbert P. Coaggregation between freshwater bacteria within biofilm and planktonic communities. *FEMS Microbiol Lett*, 220:133–140, 2003.

Rickard AH, McBain AJ, Stead AT, and Gilbert P. Shear rate moderates community diversity in freshwater biofilms. *Appl Environ Microbiol*, 70:7426–7435, 2004.

Riggio MP and Lennon A. Identification of oral *Peptostreptococcus* isolates by PCR-restriction fragment length polymorphism analysis of 16S rRNA genes. *J Clin Microbiol*, 41:4475–4479, 2003.

Rolph HJ, Lennon A, Riggio MP, Saunders WP, MacKenzie D, Coldero L, and Bagg J. Molecular identification of microorganisms from endodontic infections. *J Clin Microbiol*, 39:3282–3289, 2001.

Rosan B, Slots J, Lamont RJ, Listgarten MA, and Nelson GM. *Actinobacillus actinomycetemcomitans* fimbriae. *Oral Microbiol Immunol*, 3:58–63, 1988.

Rüdiger SG, Carlén A, Meurman JH, Kari K, and Olsson J. Dental biofilms at healthy and inflamed gingival margins. *J Clin Periodontol*, 29:524–530, 2002.

Russell C and Coulter WA. Continuous monitoring of pH and Eh in bacterial plaque grown on a tooth in an artificial mouth. *Appl Microbiol*, 29:141–144, 1975.

Sakamoto M, Suzuki M, Umeda M, and Benno Y. Reclassification of *Bacteroides forsythus* (Tanner et al. 1986) as *Tannerella forsythensis* corrig., gen. nov., comb. nov. *Int J Syst Evol Microbiol*, 52:841–849, 2002.

Sanger F, Nicklen S, and Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*, 74:5463–5467, 1977.

Sanz M, Lau L, Herrera D, Morillo JM, and Silva A. Methods of detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *J Clin Periodontol*, 31:1034–1047, 2004.

Sauer K and Camper AK. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol*, 183:6579–6589, 2001.

Sawada S, Kokeguchi S, Takashiba S, and Murayama Y. Development of 16S rDNA-based PCR assay for detecting *Centipeda periodontii* and *Selenomonas sputigena*. *Lett Appl Microbiol*, 30:423–426, 2000.

Schenkein H. The pathogenesis of periodontal diseases. *J Periodontol*, 70(4):457–470, 1999.

Schüpbach P, Osterwalder V, and Guggenheim B. Human root caries: microbiota in plaque covering sound, carious and arrested carious root surfaces. *Caries Res*, 29:382–395, 1995.

Scola BL and Raoult D. Molecular identification of *Gemella* species from three patients with endocarditis. *J Clin Microbiol*, 36:866–871, 1998.

Shah HN and Collins MD. Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. *Int. J. Syst. Bacteriol.*, 38:128–131, 1988.

Shannon CE and Weaver W. *The mathematical theory of communication.* University of Illinois press, Urbana, USA., 1963.

Shellis RP. A synthetic saliva for cultural studies of dental plaque. *Arch Oral Biol*, 23:485–489, 1978.

Shen S, Samaranayake LP, and Yip HK. *In vitro* growth, acidogenicity and cariogenicity of predominant human root caries flora. *J Dent*, 32:667–678, 2004.

Siqueira Jr. JF and Rôças IN. Nested PCR detection of *Centipeda periodontii* in primary endodontic infections. *J Endod*, 30:135–137, 2004.

Sissons CH. Artificial dental plaque biofilm model systems. *Adv Dent Res*, 11:110–126, 1997.

Slots J. Update on *Actinobacillus Actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease. *J Int Acad Periodontol*, 1:121–126, 1999.

Smith DJ. The oral cavity as an immunological entity. In J. Slots MAT, editor, *Contemporary oral microbiology and immunology*, pages 524–532. Mosby, St. Louis, 1992.

Sneath PH and Barrett SJ. A new species of *Neisseria* from the dental plaque of the domestic cow, *Neisseria dentiae* sp. nov. *Lett Appl Microbiol*, 23:355–358, 1996.

Socransky SS and Manganiello SD. The oral microbiota of man from birth to senility. *J Periodontol*, 42:485–496, 1971.

Socransky SS, Haffajee AD, and Dzink JL. Relationship of subgingival microbial complexes to clinical features at the sampled sites. *J Clin Periodontol*, 15:440–444, 1988.

Socransky SS, Haffajee AD, Cugini MA, Smith C, and Kent RL. Microbial complexes in subgingival plaque. *J Clin Periodontol*, 25:134–144, 1998.

Sorensen WP, Löe H, and Ramfjord SP. Periodontal disease in the beagle dog. *J Periodontal Res*, 15:380–389, 1980.

Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol*, 292:107–113, 2002.

Suda R, Kobayashi M, Nanba R, Iwamaru M, Hayashi Y, Lai CH, and Hasegawa K. Possible periodontal pathogens associated with clinical symptoms of periodontal disease in Japanese high school students. *J Periodontol*, 75:1084–1089, 2004.

Sukontapatipark W, el Agroudi MA, Selliseth NJ, Thunold K, and Selvig KA. Bacterial colonization associated with fixed orthodontic appliances. *Eur J Orthod*, 23:475–484, 2001.

Sundqvist G, Figdor D, Persson S, and Sjgren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 85:86–93, 1998.

Swofford DL. PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods), version 4.0b2a. Computer program published by Sinauer Associates, Sunderland, Massachusetts, USA, 1999.

Syed SA and Loesche WJ. Survival of human dental plaque flora in various transport media. *Appl Microbiol*, 24:638–644, 1972.

Syed SA, Svanberg M, and Svanberg G. The predominant cultivable dental plaque flora of beagle dogs with periodontitis. *J Clin Periodontol*, 8:45–56, 1981.

Takada K and Hirasawa M. Expression of trypsin-like activity by the genera *Corynebacterium* and *Actinomyces* in canine periodontitis. *J Med Microbiol*, 49:621–625, 2000.

Tanner AC, Haffer C, Bratthall GT, Visconti RA, and Socransky SS. A study of the bacteria associated with advancing periodontitis in man. *J Clin Periodontol*, 6:278–307, 1979.

Tanner A, Maiden MF, Macuch PJ, Murray LL, and Kent RL. Microbiota of health, gingivitis, and initial periodontitis. *J Clin Periodontol*, 25:85–98, 1998.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, and Higgins DG. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, 25:4876–4882, 1997.

van der Hoeven JS, van den Kieboom CW, and Schaeken MJ. Sulfate-reducing bacteria in the periodontal pocket. *Oral Microbiol Immunol*, 10:288–290, 1995.

Vandamme P, Falsen E, Rossau R, Hoste B, Segers P, Tytgat R, and Ley JD. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol*, 41:88–103, 1991.

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, and Smith HO. Environmental genome shotgun sequencing of the Sargasso Sea. *Science*, 304:66–74, 2004.

Vilà C, Maldonado JE, and Wayne RK. Phylogenetic relationships, evolution, and genetic diversity of the domestic dog. *J Hered*, 90:71–77, 2003.

von Graevenitz A, Pünter-Streit V, Riegel P, and Funke G. Coryneform bacteria in throat cultures of healthy individuals. *J Clin Microbiol*, 36:2087–2088, 1998.

Wade WG, Downes J, Dymock D, Hiom SJ, Weightman AJ, Dewhirst FE, Paster BJ, Tzellas N, and Coleman B. The family *Coriobacteriaceae*: reclassification of *Eubacterium exiguum* (Poco et al. 1996) and *Peptostreptococcus heliotrinreducens* (Lanigan 1976) as *Slackia exigua* gen. nov., comb. nov. and *Slackia heliotrinireducens* gen. nov., comb. nov., and *Eubacterium lentum* (Prevot 1938) as *Eggerthella lenta* gen. nov., comb. nov. *Int J Syst Bacteriol*, pages 595–600, 1999.

Wayne RK, Valkenburgh BV, and O'Brien SJ. Molecular distance and divergence time in carnivores and primates. *Mol Biol Evol*, 8:297–319, 1991.

Weinberg MA and Bral M. Laboratory animal models in periodontology. *J Clin Periodontol*, 26:335–340, 1999.

Whittaker CJ, Klier CM, and Kolenbrander PE. Mechanisms of adhesion by oral bacteria. *Annu Rev Microbiol*, 50:513–552, 1996.

Wilson M, Kpendema H, Noar JH, Hunt N, and Mordan NJ. Corrosion of intra-oral magnets in the presence and absence of biofilms of *Streptococcus sanguis*. *Biomaterials*, 16:721–725, 1995.

Wimpenny JWT, Kinniment SL, and Scourfield MA. The physiology and biochemistry of biofilm. In S. P. Denyer SPG and Sussman M, editors, *Microbial Biofilms: Formation and Control*, Society for Applied Bacteriology Technical Series 30, pages 51–94. Oxford: Blackwell Scientific, 1993.

Wimpenny J. Introduction. In Wimpenny J, editor, *CRC Handbook of Laboratory Model Systems for Microbial Ecosystems.*, pages 1–17. Boca Raton: CRC Press., 1988.

Woese CR. Bacterial evolution. *Microbiol Rev*, 51:221–271, 1987.

Wunder JA, Briner WW, and Calkins GP. Identification of the cultivable bacteria in dental plaque from the beagle dog. *J Dent Res*, 55:1097–1102, 1976.

Ye P, Harty DW, Chapple CC, Nadkarni MA, Carlo AA, and Hunter N. Streptococci and *Actinomyces* induce antibodies which cross react with epithelial antigens in periodontitis. *Clin Exp Immunol*, 131:468–476, 2003.

Zylber LJ and Jordan HV. Development of a selective medium for detection and enumeration of *Actinomyces viscosus* and *Actinomyces naeslundii* in dental plaque. *J Clin Microbiol*, 15:253–259, 1982.